While lactic acid–producing fermentation has long been used to improve the storability, palatability, and nutritive value of perishable foods, only recently have we begun to understand just why it works. Since the publication of the third edition of Lactic Acid Bacteria: Microbiological and Functional Aspects, substantial progress has been made in a number of areas of research. Completely updated, the Fourth Edition covers all the basic and applied aspects of lactic acid bacteria and bifidobacteria, from the gastrointestinal tract to the supermarket shelf.

Topics discussed in the new edition include:

• Revised taxonomy due to improved insights in genetics and new molecular biological techniques
• New discoveries related to the mechanisms of lactic acid bacterial metabolism and function
• An improved mechanistic understanding of probiotic functioning
• Applications in food and feed preparation
• Health properties of lactic acid bacteria
• The regulatory framework related to safety and efficacy

Maintaining the accessible style that made previous editions so popular, this book is ideal as an introduction to the field and as a handbook for microbiologists, food scientists, nutritionists, clinicians, and regulatory experts.
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Preface

The previous editions of this book have never looked very much like their predecessors, and this fourth edition is no exception. Due to the accumulation of new findings during the seven years that has passed since the previous update, practically all chapters are either completely rewritten or are totally new. We (the editors) and the contributors have strived to reach a proper balance between the well-established “eternal truths” and the novel and even controversial findings. While keeping the format of individual chapters as reviews, a certain compromise between comprehensiveness and readability has been aimed at in order to avoid an excessive length and too massive a size of the volume. In addition to purely scientific aspects related to lactic acid bacteria and their applications, the regulatory framework related to their safety and efficacy, particularly in probiotic use, has also been reviewed. We hope that the book will find its audience both as an introduction to the field for an advanced student and as a handbook for microbiologists, food scientists, nutritionists, clinicians, and regulatory experts. The editors are indebted to Dr. Anna Lyra for skillful and tireless help with the editorial process.

Sampo Lahtinen
Seppo Salminen
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Chapter 1

Lactic Acid Bacteria: An Introduction

Atte Von Wright and Lars Axelsson

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1.1 Background

At the turn of the 20th century the term “lactic acid bacteria” (LAB) was used to refer to “milk-souring organisms.” While similarities between milk-souring organisms and other bacteria producing lactic acid were soon observed, the monograph by Orla-Jensen (1919) formed the basis of the present classification of LAB. The criteria used by Orla-Jensen (cellular morphology, mode of glucose fermentation, temperature ranges of growth, and sugar utilization patterns) are still very important for the classification of LAB, although the advent of more modern taxonomic tools, especially molecular biological methods, have considerably increased the number of LAB genera from the four originally recognized by Orla-Jensen (\textit{Lactobacillus}, \textit{Leuconostoc}, \textit{Pediococcus}, and \textit{Streptococcus}).

LAB have traditionally been associated with food and feed fermentations, and are generally considered beneficial microorganisms, some strains even as health-promoting (probiotic) bacteria. However, some genera (\textit{Streptococcus}, \textit{Lactococcus}, \textit{Enterococcus}, \textit{Carnobacterium}) also contain species or strains that are recognized human or animal pathogens. A thorough understanding of taxonomy, metabolism, and molecular biology of LAB is thus necessary to fully utilize the technological, nutritional, and health-promoting aspects of LAB while avoiding the potential risks.

In the following sections a brief and concise overview of the present understanding of the taxonomy and physiological and metabolic characteristics of LAB are presented. The important genera and species are specifically dealt with in the other chapters of this book, and some information will, inevitably, be redundant. However, this general introduction hopefully helps the reader to familiarize with the subject and makes the digestion of the more specific aspects easier.

1.2 Current Taxonomic Position of LAB

LAB constitutes a group of gram-positive bacteria united by certain morphological, metabolic, and physiological characteristics. They are nonsporulating, nonrespiring but aerotolerant cocci or rods, which produce lactic acid as one of the main fermentation products of carbohydrates. They lack genuine catalase and are devoid of cytochromes. According to the current taxonomic classification, they belong to the phylum \textit{Firmicutes}, class \textit{Bacilli}, and order \textit{Lactobacillales}. The different families include \textit{Aerococcaceae}, \textit{Carnobacteriacea}, \textit{Enterococcaceae}, \textit{Lactobacillaceae}, \textit{Leuconostocaceae}, and \textit{Streptococcaceae} (http://www.uniprot.org/taxonomy/186826). The common genera and their main characteristics are listed in Table 1.1, and more specific taxonomic information is provided in the specific chapters devoted to these LAB groups in the subsequent sections of this book.

Phylogenetically, LAB can be clustered on the basis of molecular biological criteria, such as rRNA sequencing, and an example of a phylogenetic tree differentiating LAB from the other bacterial groups in the phylum \textit{Firmicutes} is shown in Figure 1.1 as indicated in Chapter 2 (The Genetics of Lactic Acid Bacteria) the ancestral LAB have apparently been \textit{Bacillus}-like soil organisms, which subsequently have lost several genes and the associated physiological functions while adapting to nutritionally rich ecological niches.

1.3 Carbohydrate Fermentation Patterns

1.3.1 Homo- and Heterolactic Fermentation

Because LAB do not possess a functional respiratory system, they have to obtain their energy by substrate-level phosphorylation. With hexoses there are two basic fermentative pathways. The
### Table 1.1 Common Genera of LAB and Their Differential Characteristics

<table>
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<th>Family</th>
<th>Genera</th>
<th>Shape</th>
<th>CO₂ from Glucose</th>
<th>Growth at 10°C</th>
<th>Growth at 45°C</th>
<th>Growth in 6.5% NaCl</th>
<th>Growth in 18% NaCl</th>
<th>Growth at pH 4.4</th>
<th>Growth at pH 9.6</th>
<th>Type of Lactic Acid</th>
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<td>Aerococcus</td>
<td>Cocci (tetrads)</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>L</td>
</tr>
<tr>
<td>Carnobacteriaceae</td>
<td>Carnobacterium</td>
<td>Rods</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>ND</td>
<td>−</td>
<td>ND</td>
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<td>L</td>
</tr>
<tr>
<td>Enterococcaceae</td>
<td>Enterococcus</td>
<td>Cocci</td>
<td>−</td>
<td>+</td>
<td>+</td>
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**Note:** ND, not determined.

*a* Some Weissella strains are rod shaped.

*b* In older literature lactococci are referred to as Group N streptococci.
The homofermentative pathway is based on glycolysis (or Embden–Meyerhof–Parnas pathway) and produces virtually only lactic acid (Figure 1.2a). Heterofermentative or heterolactic fermentation (also known as pentose phosphoketolase pathway, hexose monophosphate shunt, or 6-phosphogluconate pathway) produces, in addition to lactic acid, significant amounts of CO₂ and ethanol or acetate (Figure 1.2b). As a general rule, pentoses can only be fermented heterofermentatively by entering the pathway as either ribulose-5-phosphate or xylulose-5-phosphate (Kandler 1983), but then (as is obvious from the fermentation scheme outlined in Figure 1.2b) CO₂ is not produced.

Theoretically, homolactic fermentation produces 2 moles of ATP per mole of glucose consumed. In heterolactic fermentation the corresponding yield is only 1 mole of ATP if the acetyl phosphate formed as an intermediate is reduced to ethanol. However, if acetyl phosphate is converted to acetic acid in the presence of alternative electron acceptors, an extra ATP is formed.

Hexoses other than glucose (mannose, galactose, fructose) enter the major pathways outlined above after different isomerization and phosphorylation steps as either glucose-6-phosphate or fructose-6-phosphate. For galactose there are two different pathways, depending on whether it enters the cell as galactose-6-phosphate (via the so-called phosphoenolpyruvate-dependent phosphotransferase system or PEP:PTS; see Section 1.4.2.4) or as free galactose imported by a specific transport system.
permease. In the former case the tagatose phosphate pathway is employed (Figure 1.3a) (Bisset and Andersson 1974), and the so-called Leloir pathway (Figure 1.3b) in the latter (Kandler 1983).

The fermentation type is an important taxonomic criterion. The genera *Leuconostoc*, *Oenococcus*, and *Weissella* are obligate heterofermentative, as well as the so-called Group III lactobacilli (e.g., *Lactobacillus brevis*, *Lb. buchneri*, *Lb. fermentum*, and *Lb. reuteri*). Group I lactobacilli (*Lb. acidophilus*, *Lb. delbrueckii*, *Lb. helveticus*, *Lb. salivarius*), on the other hand, are obligate homofermentative (i.e., they cannot metabolize pentoses). Group II or facultatively heterofermentative lactobacilli (*Lb. casei*, *Lb. curvatus*, *Lb. plantarum*, and *Lb. sakei*) as well as most other LAB homofermentatively ferment hexoses, but also ferment pentoses. The division of lactobacilli in three groups (*Thermobacterium*, *Streptobacterium*, and *Betabacterium*) on the basis of their fermentation patterns, as suggested by Orla-Jensen (1919), is still used for pragmatic reasons, although it does not reflect the present phylogeny of the genus.

It should be noted that the outline presented in this chapter represents a generalization, for which there are exceptions, for example, the homolactic fermentation of a pentose (Tanaka et al. 2002) and the homolactic fermentation of fructose by obligate heterofermentative lactobacilli (Saier et al. 1996).

### 1.3.2 Fermentation of Disaccharides

Due to the presence of lactose in milk, the metabolism of this disaccharide has been extensively studied, especially in the species used in dairy applications. Lactose can enter the cell either by the means of a specific permease or as lactose phosphate by a lactose-specific PEP:PTS system, and in some cases both systems can coexist (Thompson 1979). If the transport is permease mediated, lactose is cleaved to glucose and galactose by β-galactosidase, and both of these monosaccharides can subsequently enter the major fermentation pathways. In the case of PEP:PTS system, another enzyme, phospho-β-D-galactosidase, is needed to split lactose phosphate to glucose and

---

**Figure 1.3** Galactose metabolism in LAB. (a) Tagatose-6-phosphate pathway. (b) Leloir pathway.
galactose-6-phosphate. Glucose is then processed by the glycolytic pathway, while galactose-6-phosphate enters the tagatose-6-phosphate pathway.

*Lactococcus lactis* typically has a lactose PEP:PTS system, while in many species, such as leuconostocs, *Streptococcus thermophilus*, and thermophilic lactobacilli, the permease system is typical (Hutkins and Morris 1987; Premi et al. 1972). In *S. thermophilus* and thermophilic lactobacilli, the galactose moiety is not metabolized but excreted into the medium.

Maltose fermentation in LAB has been extensively studied in lactococci, and in this genus the permease system seems to be operational (Sjöberg and Hahn-Hägerdahl 1989). Another well-known example is *Lb. sanfanciscensis*, a lactobacillus found in sourdoughs. This bacterium converts maltose to glucose-1-phosphate and glucose by maltose phosphorylase. Glucose-1-phosphate is used by the bacterium as an energy source, while glucose is excreted into the medium to be used by a yeast, *Candida milleri* (Stolz et al. 1993).

Sucrose fermentation is generally based on the permease system and the action of sucrose hydrolase, which splits the disaccharide to glucose and fructose. In lactococci, a sucrose-specific PEP:PTS system accompanied by sucrose-6-phosphate hydrolase also appears to be functional, producing glucose-6-phosphate and fructose (Thompson and Chassy 1981). Sucrose may also have a role in exopolysaccharide formation in certain LAB. In *Leuconostoc mesenteroides*, sucrose is cleaved by a cell wall–associated enzyme, dextranucrase, and the glucose moiety is used for dextran synthesis, while fructose is fermented in the usual manner (Cerning 1990).

### 1.3.3 Alternative Fates of Pyruvate

Pyruvate has a central role in the fermentation pathways, usually acting as an electron acceptor to form lactic acid and thus help maintain the oxidation–reduction balance in the cell. However, depending on the LAB strain and specific growth conditions, alternative pyruvate-utilizing pathways exist. They are summarized in Figure 1.4 and briefly discussed below.

#### 1.3.3.1 Diacetyl/Acetoin Pathway

The pathway(s) leading to diacetyl (butter aroma) and acetoin/2,3-butanediol is common in many LAB, but technologically important in certain dairy lactococci and leuconostocs. The pathway requires a surplus of pyruvate relative to the need of NAD+ regeneration. In milk the pyruvate surplus is provided by the breakdown of citrate, which is typically present in significant amounts (~1.5 mg/ml). The metabolism of citrate and the formation of diacetyl have been reviewed by Hugenholz (1993). In short, citrate is transported into the cell by a specific permease and cleaved by citrate lyase to yield oxaloacetate and acetate. Oxaloacetate is subsequently decarboxylated to pyruvate and CO2 by oxaloacetate decarboxylase. As can be seen in Figure 1.4, there are two alternative routes from pyruvate to diacetyl, but the one involving α-acetolactate appears to be more common since this compound is frequently detected as an intermediate. It should be noted that diacetyl formation from α-acetolactate occurs spontaneously without a specific enzymatic reaction.

#### 1.3.3.2 Pyruvate–Formate Lyase System

As a response to substrate limitation and in anaerobic conditions, LAB can resort to another branch of pyruvate metabolism by the formation of formic acid and acetyl-CoA in a reaction catalyzed by pyruvate–formate lyase (Thomas et al. 1979; Kandler 1983). The acetyl-CoA formed can
act as an electron acceptor to yield ethanol, or it can be used for substrate-level phosphorylation and subsequent ATP synthesis, giving acetate as the end product. The final metabolic end products, even in a LAB species with homolactic hexose metabolism, may thus in certain conditions be lactate, acetate, formate, and ethanol. The term “mixed acid fermentation” has been used to differentiate this phenomenon from the normal heterolactic fermentation.

### 1.3.3.3 Pyruvate Oxidase Pathway

In the presence of oxygen, pyruvate can be converted to acetate by the action of pyruvate oxidase. In this reaction \( \text{H}_2\text{O}_2 \) is also formed. In some cases this pathway may lead to the significant aerobic formation of acetic acid (Sedewitz et al. 1984).
1.3.3.4 Pyruvate Dehydrogenase Pathway

This pathway is functional particularly in lactococci (Cogan et al. 1989; Smart and Thomas 1987). Pyruvate is oxidized to acetyl-CoA, while CO₂ and NADH are formed. The reaction may have a catabolic function in providing acetyl-CoA for lipid biosynthesis (the pyruvate–formate–lyase system may have a similar role in anaerobic conditions). The usual end product, however, is acetate, and Lc. lactis has been shown to perform homoacetic fermentation in aerated cultures under substrate limitation (Smart and Thomas 1987).

1.3.4 Alternative Electron Acceptors

In standard fermentations pyruvate or acetyl-CoA and acetaldehyde act as electron acceptors to maintain the oxidation–reduction balance. However, alternative electron acceptors may be available in the cell, and these can sometimes have profound effects on the energetics and growth rate of LAB.

1.3.4.1 Oxygen as an Electron Acceptor

Although LAB are independent of oxygen, its presence is often stimulatory to the growth. An example in heterofermentative LAB is the conversion of acetyl phosphate to acetic acid. This can occur in the presence of alternative electron acceptors, oxygen being one of them. As pointed out in Section 1.3.1, this pathway provides the cell with an additional ATP in comparison to the standard fermentation resulting in ethanol formation. The effect of oxygen has been demonstrated with leuconostocs, which increase their growth rate in aerated cultures accompanied by the production of acetate instead of ethanol, indicating the presence of active NADH oxidase (Lucey and Condon 1986). This phenomenon is apparently very common among heterofermentative LAB (Borch and Molin 1989). Indeed, some heterofermentative LAB have a reduced ability to metabolize glucose anaerobically due to the lack of acetaldehyde dehydrogenase essential for the ethanol branch of the heterofermentative pathway (Eltz and Vandemark 1960; Stamer and Stoyla 1967).

In homofermentative LAB, NADH oxidases may compete with lactate dehydrogenase leading to a surplus of pyruvate, which can be shifted to the diacetyl/acetoin pathway (see Section 1.3.3.1). This effect has been demonstrated in aerated cultures of homofermentative LB (Borch and Molin 1989).

Polyols represent a class of substrates that can often be fermented only when oxygen is present. Examples include oxygen-dependent glycerol fermentation by Pediococcus pentaces (Dobrogosz and Stone 1962) and mannitol fermentation of Lb. casei (Brown and Vandemark 1968).

Some species of LAB are also able to shift from anaerobic metabolism to oxidative phosphorylation when provided with heme or hemoglobin in the growth medium. The phenomenon was described already in the 1960s and 1970s for enterococci, leuconostocs, and lactococci (Ritchey and Seeley 1976), but largely neglected until the genomic sequence of Lc. lactis IL1403 revealed the presence of genes for the synthesis of cytochrome oxidase (Bolotin et al. 2001). This lead to a “revival” of studies on respiration by lactococci (Gaudu et al. 2002), with clear technological implications (Koebmann et al. 2008).

1.3.4.2 Organic Compounds as Electron Acceptors

The fermentation patterns of heterofermentative LAB can be profoundly affected also by the presence of organic molecules able to act as electron acceptors and shift the direction from acetyl phosphate to acetate. These reactions are often referred as cofermentations.
The breakdown product of citrate, oxaloacetate, can be reduced to malate and finally to succinic acid with fumarate as an intermediate by heterofermentative LAB in cofermentation with glucose. This pathway has been demonstrated in *Lb. mucosae* (Axelsson 1990; Roos et al. 2000), and is apparently common in LAB isolated from plant material (Kaneuchi et al. 1988). Another type of citrate cofermentation has been characterized by Ramos and Santos (1996), in which the shift to acetate and lactic acid is accompanied by the accumulation of 2,3-butanediol.

Glycerol is also used as an electron acceptor by several heterofermentative LAB (Schütz and Radler 1984; Talarico and Dobrogosz 1990). Glycerol is first dehydrated to 3-hydroxypropionaldehyde, which is subsequently reduced to 1,3-propionaldehyde, which is the main fermentation product in addition to lactate, acetate, and CO₂. The intermediate, 3-hydroxypropionaldehyde, is accumulated and secreted by *Lb. reuteri* and is also known as reuterin, a potent antimicrobial substance (Axelsson et al. 1989; Talarico and Dobrogosz 1989).

The fermentation of fructose by heterofermentative LAB is an example of the same compound acting both as an electron donor and the electron acceptor with mannitol as an end product (Eltz and Vandemak 1960). While the fermentation occurs in the standard heterolactic fashion, some of the sugar is reduced by mannitol dehydrogenase. The overall balance of the fermentation is thus:

\[
3 \text{ Fructose} + 2 \text{ ADP} + 2 \text{ P}_i \rightarrow 1 \text{ lactate} + 1 \text{ acetate} + 1 \text{ CO}_2 + 2 \text{ mannitol} + 2 \text{ ATP}.
\]

### 1.4 Bioenergetics, Solute Transport, and Related Phenomena

In respiratory microorganisms ATP synthesis is intimately linked with the generation of the proton-motive force (PMF) across the cell membrane. Two factors contribute to PMF: the electrical potential (\(\Delta\psi\)) generated by the proton gradient and the pH gradient (\(\Delta\text{pH}\)) due to intracellular alkaline conditions in comparison to the extracellular environment. The energy of the inward flow of protons by PMF can be used to generate ATP by the H⁺-translocating ATP synthase (or simply ATP synthase). Normally LAB do not possess the electron transport chain, but they have a related enzyme system with a reverse activity, H⁺-dependent ATPase. This enzyme helps maintain the intracellular pH at a tolerable level by pumping protons out of the cell in an ATP-consuming reaction. This system is especially relevant in LAB such as enterococci, lactococci, and streptococci, which generally do not tolerate internal pH below 5.0 (Konings et al. 1989). Since this system deprives the cell of ATP that could be used in biosynthetic reactions, there are also alternative means to maintain PMF, mainly by the so-called energy recycling.

The PMF is crucial also in the transport of several solutes (secondary or PMF-driven symport). Other types of transport include the so-called primary transport, precursor–product antiport, and group translocation systems.

#### 1.4.1 Energy Recycling and PMF

The efflux of fermentation end products, such as lactate, can theoretically maintain PMF without consuming ATP, if the efflux is associated with proton symport (Michels et al. 1979). This means that a net charge (in the case of lactate several protons) must leave the cell together with the end product. This system has been shown to operate in *Lc. lactis* at pH > 6.3 and in low external lactate concentrations (ten Brink et al. 1983), meaning that this energy-saving process is only operating at the initial stage of growth in a batch culture. However, in ecological conditions where external lactate is either diluted away or consumed by other microorganisms, this advantage might be
considerable. Other fermentation end products may also serve for the generation of PMF. An energy-recycling system based on acetate efflux has been reported to operate in \textit{Lb. plantarum} (Tseng et al. 1991).

Malolactic fermentation (MLF) is another example of energetically advantageous generation of PMF. MLF is based on the conversion of malate to lactic acid and CO$_2$ in a reaction catalyzed by l-malate:NAD$^+$ decarboxylase (malolactic enzyme) (Kunkee 1991). This reaction is of a crucial importance in the ripening of grape wines.

It has been shown that in LAB performing MLF, the system can act as an indirect proton pump in which the precursor (malate) is exchanged for a protonated product (lactate) (Poolman et al. 1991; Salema et al. 1996). The PMF generated by MLF could be high enough for ATP synthesis by the reversal of the function of H$^+$-dependent ATPase, but generally MLF is considered rather an energy conservation process.

The benefits of citrate metabolism and the decarboxylation of amino acids (biogenic amine formation) can be explained by similar mechanisms in which negatively charged compounds are exchanged with more electroneutral products (Poolman 1993).

### 1.4.2 Solute Transport

#### 1.4.2.1 PMF-Driven Symport of Solutes

PMF-driven symport is based on specific permeases or carriers, which translocate the solute—usually a nutrient—across the membrane in symport with a proton. While some sugars are transported with the permease system, the system is particularly relevant for the transport of amino acids and dipeptides (at least in lactococci) (Konings et al. 1989; Smid et al. 1989).

#### 1.4.2.2 Primary Transport

Primary transport is also called phosphate bond linked transport. ATP-binding cassette transporters (ABC transporters), such as the oligopeptide transport system and one of the dipeptide transport systems (see Section 1.5) and transporters associated with the defense against osmotic shock and excretion of unwanted compounds, are typical examples of primary transporters (Poolman 2002).

#### 1.4.2.3 Precursor–Product Antiport

Many LAB can derive energy from imported arginine by the arginine deiminase pathway, which leads to ATP formation from carbamyl phosphate and ornithine export. It has been established in lactococci that this exchange reaction has a 1:1 stoichiometry and is mediated by a single membrane-associated arginine/ornithine antiporter (Poolman et al. 1987).

#### 1.4.2.4 Group Translocation: Phosphoenolpyruvate: Sugar Phosphotransferase System

The phosphoenolpyruvate:sugar phosphotransferase system (PEP:PTS) is a complex machinery that translocates a sugar across the membrane with simultaneous phosphorylation. This translocation is not dependent on concentration gradients, and the energy of the process is provided by the high-energy phosphate of PEP. The energy of the phosphoryl group is carried by a chain of
PTS-specific proteins to a membrane-located enzyme, which mediates the transport and phosphorylation of the sugar (for a general review, see Postma et al. 1993).

The general features of the system are outlined in Figure 1.5. The first two proteins of the cascade, enzyme I (EI) and heat-stable protein (HPr), can be shared by several PTS systems, whereas enzyme II BC (EIIBC) and enzyme IIA (EIIA) are sugar specific. The sugar-specific proteins can also exist as a single fusion protein.

Although PTS systems have also been detected in heterofermentative LAB (Saier et al. 1996), the system is generally associated with the glycolytic pathway, and the level of key compound, PEP, is controlled by the rate of glycolysis of fructose diphosphate (FDP) and inorganic phosphate acting as respective activators and repressors of pyruvate kinase (Thompson 1987).

PTS and the regulation of carbohydrate metabolism. A central feature of the regulation of carbohydrate metabolism in bacteria is the carbon catabolite repression (CCR) system, which is involved in the control of the catabolism of carbohydrates other than glucose. This system, in turn, is intimately linked with the PEP:PTS transport. CCR is based on the trans-acting catabolite control protein A (CcpA), which acts by binding to a cis-acting catabolite-responsive element (cre) associated with the promoter of the pertinent gene or operon (see the review of Fujita 2009).

In gram-positive bacteria the CCR system is controlled by the phosphorylation status of the Hpr protein of the PTS system. If the phosphorylation occurs at Ser-46, instead of the standard site His-15, the result is the formation of complex with the CcpA protein and the binding of this complex with the cre element. The phosphorylation status of Hpr in turn depends on the presence of FDP. High catabolic activity will increase the pool of FDP, which in turn stimulates the kinase activity of Hpr-kinase/phosphatase (HPrK/P), the enzyme that catalyzes the phosphorylation of Ser-46. At low levels of FDP the phosphatase activity of HPrK/P is activated, freeing the Hpr to function in its normal role in the PEP:PTS transport.

While the central role of CCR is the repression of catabolic functions, it can also activate certain genes and function in the inducer exclusion–expulsion mechanisms that also help the cell prevent the induction of unwonted catabolic pathways. The outline of the CCR system and its link to PTS is presented in Figure 1.6.

Figure 1.5 Sugar transport mediated by PEP:PTS system and relation to glycolysis. PK, pyruvate kinase. See text for details.
1.5 Nitrogen Metabolism: Proteolytic System

Many LAB appear to have only a very limited capacity to synthesize amino acids from inorganic nitrogen sources and thus depend on preformed amino acids present in the growth medium. Especially the dairy LAB rely on the proteolytic degradation of external proteins and on the uptake of the resulting peptides and amino acids. The proteolytic machinery has been extensively

Figure 1.6 Schematic representation of the central role of HPr in global regulation of carbon transport and metabolism. See text for further details. PTS, phosphoenolpyruvate:sugar phosphotransferase system.

Figure 1.7 Model of Lc. lactis proteolytic pathway. Also included is transport of di- and tripeptides and free amino acids, although their role in growth in milk is limited. PrtP, membrane-anchored proteinase; Opp, oligopeptide transport system; D, di-/tripeptide transport system(s) (it should be noted that the dipeptide transport system is an ABC transporter like Opp); A, amino acid transport system(s); M, cytoplasmic membrane.
studied in dairy lactococci, and the system has been thoroughly reviewed by several authors (Kunji et al. 1996; Savijoki et al. 2006; Liu et al. 2010).

The caseinolytic activity is based on the cell wall–associated subtilisin-like serine proteinase (PrtP). The enzyme degrades casein to oligopeptides of variable sizes. Large peptides (4–18 amino acids) are transported by an oligopeptide transport system (Opp), an ABC transporter, while di- and tripeptide transport systems exist for smaller peptides. Two di- and tripeptide transport systems have been characterized, DtpT and Dpp. DtpT is a PMF-driven system, while the Dpp system is an ABC transporter like OPP. Inside the cell, the peptides are further degraded into amino acids by intracellular peptidases. The overall schema of the proteolytic system is shown in Figure 1.7.

The distribution of the different components of the proteolytic system in different lactococcal strains and in other LAB has been recently reviewed by Liu et al. (2010).

1.6 Concluding Remarks

As further discussed in Chapter 2, LAB apparently represent an adaptation of ancient Bacillus-like soil organisms to novel, nutritionally rich ecological niches. The recent taxonomic advances as well as the accumulating genetic data have further refined the phylogenetic position of this fascinating group of bacteria.

The process of adaptation has generally meant loss of many metabolic activities. These losses are compensated by efficient fermentation systems, energy recycling, transport mechanisms, acid production, and acid tolerance, providing LAB the means to successfully compete with other microorganisms in their environment.

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Chapter 2

Genetics of Lactic Acid Bacteria

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2.1 Introduction

The early genetic research on lactic acid bacteria (LAB) was dominated by studies on plasmid-associated phenomena, especially in lactococci. Recent advances in the genomics of LAB have opened novel insights into both the evolutionary aspects of LAB and the genetic basis of their important metabolic functions. This will undoubtedly lead to an increased understanding of the role of LAB in different food applications as well as in probiotic action. While a comprehensive review of all recent findings is outside the scope of this chapter, we hope to provide the reader with an overview of the interplay between the genetics and physiology of LAB that is starting to emerge.

2.2 Comparative Genomics of LAB

At the moment (September 2010) more than 1000 complete genome sequences for bacteria have been annotated in GenBank (http://www.ncbi.nlm.nih.gov/genomes/genlist.cgi?taxid=2&type=0&name=Complete%20Bacteria). Among them are complete genome sequences of 37 strains representing 22 species of nonpathogenic LAB and four *Bifidobacterium* species. In addition, 45 complete genomic sequences of pathogenic or potentially pathogenic LAB (i.e., from *Enterococcus faecalis* [1] and genus *Streptococcus* [44 from 13 species]) have been annotated. Also 48 genome sequences from nonpathogenic LAB strains (26 species including 13 new species), 21 sequences of *Bifidobacterium* strains (11 species including 6 new species), and 83 genome sequences from pathogenic LAB (4 *Enterococcus* species including 3 new species, 9 *Streptococcus* species including 5 new species) are under draft assembly (http://www.ncbi.nlm.nih.gov/genomes/genlist.cgi?taxid=2&type=3&name=Bacterial%20Assembly%20Sequences). The available genomic information allows for a review on three selected areas for which the data have provided new insights: (i) the pan and core genomes; (ii) the evolutionary history of LAB; and (iii) adaptation to specific ecological niches.

2.2.1 Core and Pan Genome

The core genome for a species can be defined as the set of genes present in all strains of a species, while the pan genome is the sum of all genes present within the species. Tettelin and co-workers (2005) showed that a core of approximately 1800 genes were present in *Streptococcus agalactiae* based on eight complete genome sequences, while the pan genome increased for each strain sequenced toward an asymptotic value of approximately 33 genes. Similar data was found when *S. pyogenes* genome sequences were analyzed. However, when analyzing *Bacillus anthracis*, the pan genome asymptotic value quickly dropped to zero, probably reflecting that *B. anthracis* is highly clonal. Due to the low number of complete genomes within each species in the nonpathogenic LAB, only little can be said about the pan genome of these species. However, the core sequences can be determined by microarray analyses.

In *S. thermophilus*, Lefébure and Stanhope (2007) calculated a pan genome of 1898 genes based on two complete and one draft genome sequence. They calculated a core sequence of 1487 genes. When all of the complete and draft sequences of *Streptococcus* were compared, they indicated that the pan genome of *Streptococcus* genus could well be over 6000 genes, with a core of only 600 genes. Rasmussen and co-workers (2008) constructed a pan genome array of >2200 genes from *S. thermophilus*, based on three complete genome sequences and additional published genes from GenBank. By hybridizing DNA isolated from 47 strains to the array, they were able to determine a core genome of 1271 genes. However, the data indicate that adding additional strain in the analysis will still decrease the core.
Table 2.1  Nonpathogenic LAB and Bifidobacterium Strains Completely Genome Sequenced

<table>
<thead>
<tr>
<th>Genus</th>
<th>Species</th>
<th>Subspecies</th>
<th>Strain</th>
<th>Genome Size</th>
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(continued)
Table 2.1 Nonpathogenic LAB and Bifidobacterium Strains Completely Genome Sequenced (Continued)

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For *Lactobacillus casei*, Cai and co-workers (2009) showed that 1941 (73%) predicted genes out of 2678 were present in all 21 strains analyzed with *Lb. casei* ATCC 334 derived microarray hybridization analysis. In *Lactobacillus plantarum*, Siezen and co-workers (2010) have compared microarray data from a set of 42 strains from fermented foods and human origin with *Lb. plantarum* WCFS1. They found a core of 2049 genes present in all 42 strains, while 121 genes did not have any homologs in other sequenced LAB.

### 2.2.2 Evolution of LAB Genomics

The LAB belong to phylum *Firmicutes*, class *Bacilli*. The common ancestor for this class is thought to have had a coding potential for about 3000 genes, while the present LAB generally have small genomes (Table 2.1), up to 3.5 megabases (Mb), and an average coding potential for 2000 genes.
Species with the smallest genomes are those with the highest adaptation to nutritionally rich environments, while those with more versatile habitats have the largest genomes.

The genome sizes of *Bifidobacterium*, which belong to the *Actinobacteria* phylum and are characterized by a high GC% content, are relatively small, ranging from 1.9 to 2.9 Mb. The evolution toward small genomes generally results from gene loss and horizontal acquisition (Makarova and Koonin 2007), with a minor role played by gene duplication. However, important duplications of peptidase genes are seen in peptidases, for example, the pepO duplications/triplications in *Lactococcus lactis* and in the *Lb. casei* and *Lb. helveticus* (Cai et al. 2009) as well as the pepC/E duplications found in *Lb. casei* and the *Lb. delbruecki/acidophilus* group (Cai et al. 2009).

### 2.2.2.1 Gene Loss

Analysis of evolution among members of LAB suggests that pathway to *Lactobacillales* was associated with loss of about 1000 genes among those assumed for the ancestor of all *Bacilli*. This loss involved the entire set of genes involved in sporulation, some cofactor pathway genes, genes of the heme/copper-type cytochromes, as well as catalase genes. Some species (e.g., the thermophiles *Lb. delbrueckii* ssp. *bulgaricus* and *S. thermophilus*) appear, on the basis of available genomic data, to have had a particularly prominent loss of genes but also a marked number of pseudogenes, possibly related to the highly specific ecological niche inhabited (Makarova and Koonin 2007).

Some additional and differential gene loss took place during the subsequent evolution of each lineage of *Lactobacillales*, resulting in a final arrangement of their evolutionary tree consisting of five major branches with clearly separated *Lactococcus–Streptococcus* and *Enterococcus–Tetragenococcus* branches on one side, and the *Leuconostoc–Oenococcus–Weissella–Fructobacillus* branch on the other side, the branch with closely related *Pediococcus–Lb. casei* groups, and the more distant *Lb. delbrueckii* branch, including the *Lb. acidophilus* species group.

Habitat also seems to influence the speed of gene loss, as shown by the comparative genomics of *Bifidobacterium longum* strain DJO10A freshly isolated from the human intestinal tract and grown for less than 20 generations in laboratory media and the culture collection strain *Bf. longum* NCC2705 (Lee et al. 2008). As expected, high sequence identity were found, with the exception of 23 DNA regions, 17 only in DJO10A and the other 6 only in NCC2705. Seven regions of NCC2705 were suggested to have been deleted by means of a very precise gene loss process. This precise loss of genomic regions was experimentally confirmed by growing fresh intestinal isolate of *Bf. longum* DJO10A in a laboratory medium for 1000 generations; this caused two large deletions, one analogous to a lantibiotic-encoding region missing in NCC2705. The deleted region was between two IS30. Also, the second deleted region was flanked by mobile elements, suggesting a key role of these elements in genome deletions, which may occur in a very rapid manner (two genome deletions per 1000 generations).

### 2.2.2.2 Gene Acquisition

LAB have acquired new gene families by gene duplications and by horizontal gene transfer (HGT). The roles of conjugation, transformation, transduction, and insertion (IS) elements in HGT are discussed in more details in Sections 2.4 and 2.5. While all these mechanisms have undoubtedly contributed to the evolution of LAB genomes, the events associated with transduction and the IS-mediated genetic rearrangements are sometimes discernible from the genetic data. The available data suggest that adaptation to nutrient-rich environments was the major driving force for these gene acquisitions.
HGT is a strong driving force of gene addition. Among the 86 acquired genes that are inferred to have further shaped the common ancestor of Lactobacillales, only two do not have orthologs outside Lactobacillales. Genome analysis of one Bifidobacterium strain has shown that about 5% of genome content seems to have been recently acquired by HGT (Makarova et al. 2006).

An example of paralogous gene acquisition in LAB is provided by the presence of two paralogs of the glycolytic enzyme enolase in lactobacilli. Phylogenetic analysis show that one of the enolases in Lactobacillales is the ancestral version present in virtually all Firmicutes, while the latter has apparently been acquired by some ancestor of rod-shaped LAB, possibly by horizontal transfer from Actinobacteria.

An additional gene acquisition process is illustrated by the family of enzymes involved in peptidoglycan biosynthesis. The gene encoding the UDP-N-acetylmuramyl tripeptide synthase (murE) is present in the majority of Bacilli; however, in lactobacilli (with the exception of Lb. plantarum) a paralog of this enzyme, possibly derived from Actinobacteria, has replaced the ancestral gene (Makarova and Koonin 2007).

Evidence of HGT is also provided by the presence of a coenzyme B12 gene cluster (Santos et al. 2008) in Lb. reuteri CRL1098 encoding the complete de novo biosynthesis. Comparative genomics analysis revealed a close similarity to genes coding for the anaerobic coenzyme B12 pathways in members of the genera Listeria and Salmonella. This similarity suggests HGT of the B12 biosynthetic genes, while G+C content (36% vs. 39% of the whole genome) and codon adaptation index analysis indicate that the postulated transfer of these genes was not a recent event.

Genes providing antibiotic resistance in other bacteria have been found in antibiotic-sensitive lactobacilli, where they are associated with phenotypes unrelated to drug resistance. An example is a paralog of class II lysyl-tRNA synthetase, generally implicated in oxacillin-like antibiotic resistance, which in lactobacilli is fused to a membrane-associated domain and is probably involved in cell wall biosynthesis.

The role of phage/prophage activity in LAB gene gain is illustrated by the observation that 11 genes present in both Lb. johnsonii and Lc. lactis subsp. cremoris are localized near the IS site of a prophage, suggesting transduction events in the evolutionary history of these species. An additional example of a phage-mediated HGT process that occurs in lactobacilli is provided by the phage-defense mechanism present in both Lb. delbrueckii and Lb. casei and based on a peculiar exonuclease.

Transduction has been indicated to be part of HGT also in Actinobacteriaceae. The noninducible prophage-like element present in Bf. breve UCC 2003 harbors a 20 kb composite mobile element inserted into prophage-like sequences. A gene coding for an extracellular β-glucosidase was shown to be transcribed, while the prophage genes were silent (Ventura et al. 2005).

All LAB genomic sequences are characterized by high numbers of IS sequences, indicating their central role in genomic evolution. Lb. helveticus represents a good example of genome plasticity mediated by an extensive presence of IS (Kaleta et al. 2010). The diversification of Lb. helveticus from a common Lb. acidophilus-like gut-inhabiting ancestor has been shown to be based not only on selective gene loss but also acquisition of a large number (>200) of IS elements. A genomic survey, based on microarray analysis of 10 strains, showed the presence of 16 clusters of open reading frames (ORFs) flanked by IS elements. Four IS-flanked clusters of ORFs were shown to encode restriction/modification systems, which may have accelerated the evolution of strains challenged by phage attack in the dairy ecosystem.

ISs have a role in gene transfer also in Actinobacteriaceae. Bf. longum NCC2705 and Bf. breve UCC2003 harbors two regions related to polysaccharide biosynthesis showing strong divergence in GC%. These are flanked by IS elements suggesting an inter- and/or intraspecies transfer (Ventura et al. 2005).
2.2.3 Niche-Specific Adaptation

This section focuses on the specific cases of niche-specific adaptation of LAB with a special attention paid to the intestinal tract and the dairy environment. It should be noted that the same habitat can be shared by organisms with different evolutionary background. As an example, human intestinal bacteria *Bf. longum* and *Lb. gasseri* have de novo pathways for nucleotide biosynthesis; however, this is not the case with *Lb. johnsonii*, which also lives in the human gut (O’Sullivan et al. 2009).

2.2.3.1 Adaptation to Intestinal Environment

Soil and plants being the hypothetical first niche of the ancestral LAB makes it natural to assume that the second possible habitat could be the gut of plant-eating animals. Three major areas of genomic adaptations (Lebeer et al. 2008) are needed for a soil/plant bacterium to survive and multiply in the gut of animals: (i) resistance to host colonization barriers (i.e., low gastric pH and bile salts in the gut); (ii) adhesion to intestinal tissues to overcome the intestinal flux; and (iii) the ability to ferment some substrates more efficiently than competing bacteria.

2.2.3.1.1 Resistance to Intestinal Stress Factors

The membrane lipid composition is affected by low pH and bile salts, and in some *Lactobacillus* strains genetic factors associated with these changes have been identified. Microarray analysis has shown the induction of expression of a putative phosphatidyl glycerophosphatase in *Lb. reuteri* ATCC 55730 after an acid shock, while an increased acid sensitivity was reported by Klaenhammer and colleagues (2005) after the inactivation of a similar gene in one *Lb. acidophilus* strain. Similar observations related to a putative esterase gene of *Lb. reuteri* ATCC 55730 have been reported, and the gene is apparently also involved in bile resistance.

d-Alanine esters of teichoic acids have been proposed to have a role in cell integrity at low pH and in the presence of bile (Bron et al. 2006); DNA microarray studies identified a bile-inducible *dlt* operon in *Lb. plantarum* WCFS1. A *dltD* knocked-out mutant of *Lb. rhamnosus* GG was shown to have a reduced tolerance to simulated gastric juice challenge (Perea Veléz et al. 2007). However, the inactivation of *dltA* in *Lb. reuteri* 100-23, a rodent commensal, did not affect the in vitro viability of this strain at low pH (Walter et al. 2007).

A multidrug resistance (MDR) transporter as efflux systems for bile has been identified in *Lb. acidophilus* NCFM (Pfeiler and Klaenhammer 2009), while in *Lb. plantarum* WCFS1 Bron and co-workers (2006) identified three potential bile-pumping proteins, including a putative MDR transporter gene. Also in *Lb. reuteri* ATCC 55730, two bile-inducible MDRs have been identified, and mutations in these genes prevent the growth but not the viability of the bacterium in the presence of bile (Whitehead et al. 2008).

Bile salt hydrolases (BSHs) are able to cleave the amide bond between the steroid moiety and the amino acid side chain, and BSH activity is characteristic to species inhabiting the gut but not to LAB isolated from vegetables or dairy products. However, their role in the survival in the small intestine is apparently case specific. A BSH-deficient mutant of *Lb. plantarum* WCFS1 has
a reduced tolerance to glycine-conjugated bile salts (Lambert et al. 2008), but inactivation of two BSH genes in *Lb. acidophilus* NCFM does not affect bile tolerance (McAuliffe et al. 2005) and a triple-knockout mutant of *Lb. johnsonii* NCC533 retains its full capacity to persist in the murine intestine (Denou et al. 2008).

Bile and acid exposure can induce DNA damage. In *Lb. reuteri* ATCC 55730, the expression of *dps* (DNA protection during starvation) was stimulated by the presence of bile. However, disruption of *dps* did not affect the bile resistance of *Lb. reuteri* (Whitehead et al. 2008).

### 2.2.3.1.2 Adhesion

Another adaptation to the gut as a specific ecological niche is the adhesion to intestinal mucus/cells. Peristaltic flow is likely to induce the washout of bacterial cells from the gut. Some exogenously applied bacteria are able to temporarily colonize the intestine, a property assumed to be associated with a good adherence capacity and a desirable trait for probiotic bacteria, and also have a role in pathogen exclusion and in the interactions with host cells for immune modulation. The genomic background of the structures mediating adhesion, confirmed by means of mutant analysis, has in some cases been elucidated.

Comparative genomics with *Lb. rhamnosus* GG, a strain with a well-demonstrated capacity to persist in the human gut, has shown the presence of genes for three secreted LPXTG-like pilins (spaCBA) in this strain but not in a nonpersistent *Lb. rhamnosus* LC705. Using anti-SpaC antibodies and immunogold electron microscopy, it was possible to localize SpaC pilin at the top of the pilus. Inactivation of the *spaC* gene as well as treatment with SpaC antiserum abolished the adherence of GG to human intestinal mucus (Kankainen et al. 2009).

A mannose-specific adhesin, associated with the yeast agglutination phenotype and previously shown to be related to the mannose-specific adherence of *Lb. plantarum* to human intestinal epithelial cells, was identified in *Lb. plantarum* WCFS1 by means of a microarray-based genotyping (Pretzer et al. 2005) of a number of *Lb. plantarum* strains. The adhesin was identified as a sortase-dependent protein (SDP) and the gene was named as *msa*.

In *Lb. acidophilus* NCFM, inactivation of three surface-related proteins was shown to reduce the ability of this strain to adhere to Caco-2 cells in vitro, suggesting that in this strain adhesion is determined by multiple factors. Similar results were obtained by genome analysis of *Lb. johnsonii* NCC533, which revealed a high number of SDP genes as well as a predicted fimbrial operon (for a review, see Lebeer et al. 2008).

### 2.2.3.1.3 Nutritional Adaptation

Adaptation to the nutritional environment provided by the gut is an additional important competitive advantage. Since simple sugars are readily utilized by the host, bacteria trying to inhabit the small intestine must be provided with very efficient transport systems. On the other hand, diet and host mucins provide a large amount of complex carbohydrates reaching the colon. Bacteria with numerous genes involved in polysaccharide degradation are therefore probably better equipped to multiply in the lower part of the gut.

Comparative genome hybridization and microarray analysis of genes of *Lb. johnsonii* type strain ATCC 33200 and a strain persisting in the mouse intestine, NCC533 (Denou et al. 2008), revealed three gene loci that were both specific to NCC533 and expressed in vivo in the jejunum of NCC533-monoassociated mice. One locus was annotated as involved in mannose utilization, and its deletion resulted in an impaired ability to persist in the murine gut. NCC533 was shown to
be metabolically inactive in the colon while in the jejunum mRNAs for 297 genes were detected, including three jejunum-specific sugar phosphotransferase (PTS) transporters (fructose, glucose, and cellobiose), indicating an important role for sugar transporters in the upper part of the gut.

*L. acidophilus*, a species closely related to *L. johnsonii*, appears to have a severely reduced potential to synthesize most amino acids, vitamins, and cofactors but have an abundance of transport systems, including genes encoding peptidases and proteases. Genes present in *L. acidophilus* NCFM confer to this strain the ability to ferment many sugars, from monosaccharides to raffinose and fructooligosaccharides (FOS). FOS fermentation genes form a specific operon (Barrangou et al. 2006). The exceptional sugar fermentation ability of this strain suggests a competitive advantage not only in the small intestine but also in the colon.

In gut colonization trials comparing the cellobiose-utilizing *L. plantarum* WCFS1 and a knockout mutant, the wild type had markedly higher (100- to 1000-fold) intestinal counts in murine gut than the mutant (Bron et al. 2007). Similar evidence derived from comparative genomics is available for bifidobacteria (Ventura et al. 2007a). The genes present in a fresh gut isolate of *B. longum* (strain DJO10A) and absent in a culture collection strain (NCC2705) were mainly related to carbohydrate metabolism, and particularly to utilization of oligosaccharides, which are the most relevant carbohydrates available in the colon for bacteria.

In general, the colon-specific colonization ability of bifidobacteria has been related to their capacity to metabolize complex carbohydrates, as indicated by a number of genes for complex sugar metabolism, which, in *B. breve* and *B. longum*, account for more than 8% of the annotated genes of both species. The large majority of these hydrolases are intracellular, and their genes are usually coupled with genes encoding for the uptake of carbohydrates. About 10% of the total bifidobacterial gene content is dedicated to sugar transporters, mainly of the ABC type.

Bifidobacteria have also been shown to be able to utilize carbohydrates associated with mucin and glycosphingolipids (Ventura et al. 2007b). This relationship between the mammalian host supplying carbohydrate substrates and the commensal intestine-adapted bacteria resembles symbiosis.

### 2.2.3.2 Adaptation to Dairy Environment

LAB are associated with the production of practically all fermented dairy foods. Some of the species commercially used as starters or naturally occurring in cheese are phylogenetically closely related to species totally unable to grow in milk. The ability to ferment lactose and to degrade milk proteins (proteolysis) are the major adaptations to dairy environment by LAB.

The first studies published about molecular genetics of LAB demonstrated the plasmid location of genes involved in lactose utilization in mesophilic lactic cocci and rods. Thermophilic LAB rods were shown to have at least part of their caseinolytic ability coded by plasmids, while lactose utilization is chromosomally encoded (Morelli et al. 2004). Up to now the different localization of these genes, relevant for growing in milk, has not been further investigated by means of genomic studies.

An interesting comparison has been made between the dairy-associated *L. helveticus* DPC4571 and the intestinal strain *L. acidophilus* NCFM. These bacteria share 98.4% 16S ribosomal RNA identity, suggesting a close phylogenetic similarity despite extremely different niches. The analysis of completed genomes show that 75% of predicted DPC4571 ORFs have orthologs in *L. acidophilus* NCFM genome (O’Sullivan et al. 2009). However, the dairy adapted strain had 123 (IS elements not included) genes not present in the gut-adapted strain, while genes unique in the intestinal NCFM strain were 503. Among the potential 626 niche-specific genes, those involved in proteolysis and in phage resistance such as restriction/modification systems, turned out to be dairy
specific. While the milk specificity of the first set of genes is obvious, the second one is surprising and could be possibly related to the commercial use of this strain.

A comparative genomics analysis of the proteolytic system (proteinases, peptide transporters, and peptidases) in 22 sequenced LAB strains (Liu et al. 2010) has indicated genes for enzymes essential for a rapid growth in milk in several species, such as the cell wall–bound proteinase (PrtP), present in the chromosome of *Lb. acidophilus*, *Lb. johnsonii*, *Lb. bulgaricus*, *Lb. casei*, *Lb. rhamnosus*, and one strain of *S. thermophilus*, as well as on the plasmid of *Lc. lactis* subsp. *cremoris* SK11. A comparative analysis of 39 strains of *Lc. lactis* showed that the presence several peptidases, such as PepC, PepN, PepM, PepA, PepD, PepV, PepT, PepP, PepQ, DtpT, and most members of the Dpp system, is shared by almost all strains, the plant-derived strains harboring the largest set of proteolytic genes. In other species of LAB, the situation is reversed. Plant-associated species such as *Lb. plantarum*, *Oenococcus oeni*, and *Leuconostoc mesenteroides*, encode less proteolytic enzymes, which agrees with their ecological niche that is rich in fiber but contains less proteins. That the PepE/PepG (endopeptidases) and PepI/PepR/PepL (proline peptidases) superfamilies of proteolytic systems are absent from coccoid LAB but present in LAB rods appears to be one of the differentiating factors between the two groups.

One of the aspects of the well-known cooperation observed between *Lb. delbrueckii* ssp. *bulgaricus* and *S. thermophilus* used to ferment yogurt has been clarified by comparison of their proteolytic systems. *Lb. delbrueckii* ssp. *bulgaricus* was found to have the Dpp system (Liu et al. 2010) with a preference for hydrophobic di-/tripeptides complementing *S. thermophilus* transport activity based on the general di-/tripeptide transporter DtpT, meaning that more peptides can be utilized by both bacteria when grown together. An additional and fascinating evidence of coadaptation to dairy environment by the two bacterial species is provided by the HGT events that have possibly occurred between them as indicated by in silico analysis. It appears that the cocci have transferred to the rods the ability to produce EPS, while *S. thermophilus* received from *Lb. delbrueckii* ssp. *bulgaricus* the gene cluster cbs-cblB(cglB)-cysE, coding for the metabolism of sulfur-containing amino acids (Liu et al. 2009).

Strains of *Lb. helveticus* (Liu et al. 2010) have been shown to own an extensive panoply of proteolytic enzymes, enabling the use of this thermophilic species as a proteolytic cheese adjunct culture for degradation of bitter peptides in cheeses manufactured using mesophilic starter cultures. The extensive dairy adaptation of this species is further illustrated by the original niche of this bacterium, which is proposed to be the intestine. A crucial role in this adaptation process was played by the IS elements, accounting for the selective loss of genes associated with gut epithelium and mucus binding as well as glucosidases and PTS system genes from the ancestral *Lb. acidophilus* strains compared to *Lb. acidophilus*.

Two of the most significant niche adaptations have been seen within the *Lb. casei* (Cai et al. 2009) and the *Lc. lactis* species (Siezen and Bachmann 2008; Siezen et al. 2010). By multilocus sequence typing it was shown that *Lb. casei* approximately 1.5 million years ago separated into two main clusters, with a more recent separation of many cheese isolates approximately 10,000 years ago. In comparative genome hybridization it was shown that five out of seven cheese isolates from Denmark, Australia, and the United States had lost approximately 15–20% of the genes present in the cheese isolate ATCC 334. The genes lost are hypothetical proteins, phage genes, but also genes involved in carbohydrate metabolism and amino acid transport, and restriction/modification. Particularly a 129 kb region from 2.6 to 2.7 Mb coding for approximately 15% of all carbohydrate-utilizing genes has been lost.

The comparative genome sequence analysis of the plant isolate *Lc. lactis* KF147 also revealed that dairy lactococci (*lactis* and *cremoris* subspecies) have undergone dramatic niche adaptation
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(Lc. lactis KF147 and IL1403 have very similar nucleotide and gene order. However, IL1403 has lost gene clusters involved in plant cell wall degradation and uptake and conversion systems for such degradation products. Of special interest is a missing 51 kb conjugative transposon coding for α-glucosides. This region is readily lost when KF147 is grown in milk (Bachmann et al. 2009; Siezen et al. 2010).

2.3 Plasmid Biology of LAB

The observations by L.L. McKay about the spontaneous and acriflavine-induced loss of lactose fermentation ability of lactococcal strains suggested involvement of plasmids in this phenotype (McKay et al. 1972). Subsequent demonstration of extrachromosomal DNA in the lactococci soon led to the identification of several metabolic plasmids and their functions, such as carbohydrate fermentation and protease plasmids (Horng et al. 1991; Frère et al. 1993), citrate permease plasmids (Jahns et al. 1991) and phage resistance plasmids (Lucey et al. 1993; Gravesen et al. 1995), plasmids associated with mucoidness or ropiness (van Kranenburg et al. 1997), and bacteriocin production (Davey 1984). Several antibiotic resistance plasmids from different LAB have also been characterized (Teuber et al. 1999; Vescovo et al. 1982; Clewell et al. 1974). While metabolic plasmids are particularly typical to lactococci, they occur frequently also in other groups of LAB. While the metabolic plasmids are generally large with typical sizes of tens of kilobase pairs, the occurrence of small cryptic plasmids is very common, and they are also commonly used as replicons in vector construction. Complete plasmid sequences from many nonpathogenic and pathogenic LAB have been annotated (http://www.ncbi.nlm.nih.gov/genomes/genlist.cgi?taxid=2&type=0&name=Complete%20Bacteria).

In the course of years the study of LAB-associated plasmids has revealed many aspects that also have wider relevance in general plasmid biology. These include replication mechanisms, copy number control, and incompatibility.

2.3.1 Physical Structure, Replication Mechanisms, Host Range, and Incompatibility

2.3.1.1 Circular Plasmids

The majority of LAB-associated plasmids belong to the standard type of covalently closed circular, autonomously replicating DNA molecules replicating by two basic mechanisms: the rolling circle type of replication or the so-called theta replication.

Small rolling cycle plasmids are very typical in gram-positive bacteria, and their basic features have been reviewed by Gruss and Ehrlich (1989) and by Meijer and co-workers (1998). In this type of replication a nick is first formed by the replication initiation protein (IP) in the so-called plus-origin of replication, which is situated in the immediate vicinity of the IP gene. After the nick a multiprotein replisome starts the synthesis of a new strand while displacing the plus-strand (Figure 2.1). When a cycle has been completed, a double-stranded plasmid and a circular single-stranded intermediate is formed by cutting and religating the replaced minus-strand. A new round of DNA synthesis starts from the minus-origin of this intermediate, leading to the formation of another double-stranded plasmid.

The small lactococcal plasmids pVW01 and pSH71, which have been used in cloning vector construction, are typical examples of plasmids replicating by a rolling circle mechanism. These
plasmids have a broad host range replicating in several other gram-positive hosts and also in *Escherichia coli* (Kok et al. 1984).

The theta-replicating plasmids in LAB are generally of a medium or large size, the previously mentioned metabolic plasmids being illustrative examples. Theta replication is based on a uni- or bidirectional progressive replication fork. No extended single-stranded regions are formed. The sequences of many theta-replicating plasmids share a remarkable degree of homology in their respective replication regions. The replication protein gene (*repB*) is preceded by an AT-rich origin of replication (*repA*). One of the striking features is the presence of three successive complete and one incomplete 22 bp direct repeats with a general consensus sequence of TATANNNNN(A/T)-NAAAAA(A/T)C(T/G)(G/A)TC immediately before the promoter of the *repB* gene. Two inverted repeats, one of them between the –10 region and the start of *repB*, are also regularly found, as well as two AT-rich short (9–10 bp) repeats further upstream of the 22 bp sequences.

The lactococcal family of theta-replicating plasmids also seems to be generally compatible with each other. After screening of 12 theta-replicating plasmids, two incompatible pairs—pFV1001 and pFV1201, and pJW565 and pFW094—were found (Gravesen et al. 1997). The incompatibility region could be tentatively located within the above-mentioned region of 22 bp direct repeats and the first inverted repeat.

### 2.3.1.2 Megaplasmids

The presence of megaplasmids (size range 120–490 kbp) has been established in strains of *Lb. salivarius* and other lactobacilli (Li et al. 2007). These plasmids can be either circular or linear, the former replicating with the *repA*-based system. The mode of replication of the linear
plasmids is not well characterized, and presumably some kind of telomere structures must be present. Characteristics associated with a circular *Lb. salivarius* megaplasmids include carbohydrate metabolism and bacteriocin production.

### 2.4 Gene Transfer in LAB

The importance of HGT in the evolution of LAB genomes has already been indicated above. HGT is not a phenomenon of distant evolutionary past, but occurs continuously. Horizontal transfer of genetic material between bacteria may occur via three different mechanisms: transformation, conjugation, or transduction.

#### 2.4.1 Transformation

During natural genetic transformation the DNA released from a bacterial cell is taken up by another and is incorporated by homologous recombination into its genome. Moreover bacteria need to reach a particular physiological condition known as the competence state to receive exogenous DNA. The development of competence has been thoroughly investigated in *S. pneumoniae* and *S. mutants*, both LAB species, in which transformation is known to occur naturally. The development of competence in these two species relies on an early and a late step. The early step involves five different genes (*comABCDE*). An active peptide, derived from a precursor encoded by the *comC* gene, is responsible for the induction of the competence state after its maturation and secretion in the extracellular medium through an ABC-type transporter. Production of active peptide is positively correlated to cell density and to the presence of stressing signals in the cell growth environment (Perry et al. 2009; Prudhomme et al. 2006). This mature peptide act on gene *comD* coding for a histidine kinase able to phosphorylate regulator ComE that in this form positively regulates genes *comABCDE* and *comX*. This last gene is associated with an RNA polymerase core that can recognize the promoter region of the late genes involved in the competence activation. The late genes are the actual genes responsible for the positive result of the transformation event since they are involved in foreign DNA uptake, and in the protection of the single-strand DNA and its homologous integration into the host cell genome.

*S. thermophilus* is a very important species in food industry since it is involved in yogurt and cheese production, and for this reason researchers are focused on finding a natural DNA transfer system as a tool for improving the properties of industrial starter strains by the construction of food-grade mutants.

Comparative genomics have shown the presence of the *comX* gene and of all the genes considered crucial for the acquisition of competence in *S. thermophilus* species (Hols et al. 2005). Blomqvist and co-workers (2006) described a rapid and efficient system for natural transformation of *S. thermophilus* LMG 18311 strain. Authors obtained an artificial overexpression of gene *comX* responsible to induce the late phase of competence in the *S. thermophilus* cells. This overexpression occurs in particular cultural conditions (Todd-Hewitt broth medium) in the early logarithmic phase. In LMG 18311 strain, *comX* and *comEC* were essential for transformation and *comX* was required for controlling *comEC* expression. A later work using strain LMD-9 confirmed these findings (Gardan et al. 2009). Mutants carrying a deletion of *comX* or *comEC* genes were not transformable. Moreover, the Ami transporter controls competence by regulating *comX* transcription and the late genes involved in competence development. Strain LMD-9 was not transformable in M17 medium. However, competence was induced in a chemically defined peptide-free medium,
although the same medium was unable to induce competence in LMG 18311 and CNRZ 1066 strains. These results are in contrast with those obtained with *S. pyogeniae*, *S. gordonii*, and *S. mutans* showing competence development in rich media.

A very recent work proposed a model for competence development in *S. thermophilus* in a defined medium (Fontaine et al. 2010). A peptide ComS is produced and subsequently maturated and secreted by an AmiA3 substrate binding protein. The mature peptide is imported by an Ami transporter and interacts with a regulator that is activated. This activated regulator binds to the operator sequence of comS and comX.

The competence genes are present in the sequenced lactococcal genomes (Wydau et al. 2006) and some early reports indicated transformation to occur (Møller-Madsen and Jensen 1962). However, natural transformation is not prevalent in the lactococci at least in laboratory conditions. No information is currently available about possible competence development in *Lactobacillus* species.

### 2.4.2 Conjugation

Conjugation, or DNA transfer from a donor to a recipient by a direct cellular contact, represents an alternative approach for the introduction of DNA into poorly transformable or not transformable LAB (Thompson et al. 2001), and is also a natural way for *in vivo* HGT. Spreading of antibiotic resistance among bacteria has received great attention in recent years, and the presence of antibiotic-resistant species in the environment, especially in food products, has been copiously documented. Conjugation is considered the main DNA transfer system responsible for antibiotic resistance spread in bacteria (de la Cruz and Davies 2000) since many antibiotic resistance genes are located on mobile genetic elements. Conjugative transposons (see Section 2.5) can represent an important vehicle for spreading of antimicrobial resistance (Devirgiliis et al. 2009) since these elements can move from the genome of a donor bacterial cell to that of a recipient by conjugation. Moreover, conjugation can involve conjugative plasmids (van Kranenburg et al. 2005; Feld et al. 2008), or allows comobilization of a nonconjugative plasmid by the transfer of a conjugative one.

LAB used for food production or administrated as probiotics for promoting health in humans and animals are potential reservoir of antibiotic resistance genes and good candidates for horizontal transfer of antibiotic resistance since they are used and ingested alive and they have the opportunity to closely make contact with other organisms in different natural niches. Moreover, LAB harbor mobile genetic elements, such as plasmids and conjugative transposons, involved in HGT.

Conjugation is often associated with special sex factors, which are responsible for a high frequency of recombination. The sex factors of *Lc. lactis* 712 has been thoroughly analyzed (Gasson et al. 1995; Shearman et al. 1996). In addition to genes involved in the actual DNA transfer, the element contains a group II intron and a gene causing a “clumping” phenotype (*cluA*) associated with the high incidence of conjugation. The sex factor is also associated with an *ISSI* type IS element enabling its change of location from chromosome to plasmids.

Conjugation experiments are often carried out *in vitro*, using, in most cases, the filter mating method (Feld et al. 2008; Ito et al. 2009; Devirgiliis et al. 2009), although these *in vitro* experiments do not reproduce natural conjugation conditions, and for this reason the results obtained are not comparable to those obtained using the *in vivo* models. Recently Toomey and co-workers (2009) demonstrated the transfer of resistance determinants between LAB using two *in vivo* models, an alfalfa sprout plant and an animal rumen model. *In vivo* transfer between LAB has previously been shown only in the gastrointestinal tracts of gnotobiotic rats (Jacobsen et al. 2007) and mice (Feld et al. 2008). Feld and collaborators evaluated the transferability of a *Lb. plantarum* erythromycin resistance plasmid *in vivo* and *in vitro*. Results obtained in gnotobiotic mice
indicated that gastrointestinal tract conditions are more favorable for antibiotic resistance transfer than that obtained by filter mating. The same authors described an effect due to the indigenous gut microbiota that reduce the number of detectable transfer events. On the contrary, antibiotic treatment induced transfer of plasmids encoding for antibiotic resistance genes (Feld et al. 2008).

However, knowledge about HGT in the natural environment is still limited. Results obtained in vivo have to be evaluated, taking into consideration the effects due to the indigenous microbiota present in the gastrointestinal tract, the unfavorable intestinal environment itself, as well as the experimental conditions. In fact during the in vivo studies of antibiotic transferability, the intake of food products containing the resistant bacteria is higher than those occurring in a normal diet.

### 2.4.3 Transduction

Another DNA transfer method is transduction, mediated by bacteriophages and involving chromosomal portion or plasmids. Transduction has been infrequently described in studies on gene transfer in lactobacilli compared with transformation and conjugation. High-frequency plasmid transduction mediated by pac-type bacteriophages has been described in *Lb. delbrueckii* subsp. *lactis* and subsp. *bulgaricus* strains (Ravin et al. 2006). Plasmid transduction with three cos-type *S. thermophilus* phages beyond the genus into *Lc. lactis* has recently been described for the first time (Ammann et al. 2008). The authors ascribed the phenomenon to the high levels of similarity between genes of *S. thermophilus* and *Lc. lactis* phages.

### 2.5 IS Sequences, Transposons, and Introns

Different transposable genetic elements are important mechanisms to enhance the genetic mobility and elasticity of bacteria. IS sequences represent the simplest form of transposable genetic elements. They have a size range of 750–2000 bp and typically contain only the genes necessary for the transposition flanked by short inverted repeats. Numerous IS elements have been characterized, and their characteristics and occurrence in various bacterial groups, including lactococci, lactobacilli, enterococci, leuconostocs, and pediococci, have been extensively reviewed (Mahillon and Chandler 1998). In contrast to simple IS elements, typical transposons contain additional genes, such as antibiotic resistance determinants or genes involved in conjugative gene transfer, flanked by IS sequences. Conjugative transposons are also called integrative and conjugative elements (ICEs).

#### 2.5.1 IS Elements and Transposons

The first IS element in LAB, ISL1, was identified in *Lb. casei* by Shimizu-Kadota and co-workers (1985), and subsequently they have been shown to be universally present in LAB, as shown by the accumulating genome sequence information.

The lactococcal nisin–sucrose gene block is a well-known example of a conjugative transposon. The block is flanked by direct repeats of TTTTG (Rauch and de Vos 1992), and the conjugative nature of the nisin–sucrose transposon allows for its introduction even to heterologous hosts. Other examples of integrative and conjugative elements in LAB include ICESt1 and ICESt3 in *S. thermophilus*, which integrate site specifically into the gene coding for putative fructose-1,6-diphosphate aldolase (Bellanger et al. 2009). The proteins associated with the transfer functions of these elements share homology with corresponding proteins of a well-known enterococcal transposon, Tn916.
2.5.2 Group II Introns

Type II introns represent another type of bacterial integrative genetic elements. Introns are sequences interrupting functional genes. They are transcribed to RNA along with the rest of the gene, but subsequently spliced during the processing of mRNA. While introns are common in eukaryotes, their presence in bacteria was a relatively recent finding. Thus far, among LAB, type II introns have been detected in lactococci (Martinez-Abarca and Toro 2000) and they are regularly associated with conjugative elements or sex factors (Dunny and McKay 1999). Typical to group II introns is the formation of a closed circular structure ("lariat") during the splicing event, when the intron RNA is excised from the mRNA.

Retrohoming is also a feature of group II introns. Because the specificity of retrohoming depends on the sequences of exon binding sites, the intron can be retargeted by modifying these sequences. An example of the application of this technique is the targeted insertion of Ll.ltrB into genes coding for malate decarboxylase and tetracycline resistance in the \textit{Lc. lactis} genome (Frazier et al. 2003).

2.6 Recombinant DNA Techniques and Their Applications

The presence of plasmids and the availability of both \textit{in vivo} and \textit{in vitro} gene transfer systems, particularly electroporation (Holo and Nes 1995), have made the vector construction and recombinant DNA techniques feasible for many species and strains of LAB. Several types of cloning vectors, based on cryptic plasmids of LAB, have been constructed (for a review, see Shareck et al. 2004). Both integration and expression vectors have been developed, the expression system regulated by the lantibiotic bacteriocin nisin being one of the most successful (Mierau and Kleerebezem 2005). This system (NICE) is based on the autoregulation of nisin biosynthesis, and the host strain is first engineered to harbor the key elements of the regulatory system, \textit{nisK} and \textit{nisR}. The cloning vectors contain the promoter of the nisin structural gene (\textit{nisA}), which controls the expression of the inserted gene. Externally added nisin causes a NisR-mediated activation of the promoter and gene expression.

Although food-grade selection markers based, for example, on bacteriocin or heavy metal resistance (Takala and Saris 2002; Duan et al. 1996; Liu et al. 1996) or complementation of metabolic deficiencies (Posno et al. 1991) have been developed, genetically modified LAB have thus far not been introduced for food processes or products, apparently partly because of ambiguous consumer attitudes. Instead, a promising field of application could be the use of genetically modified LAB for therapeutic purposes, as live vaccines or production hosts for medically important proteins (Bahey el-Din et al. 2010; Wells and Mercenier 2008).

2.7 Conclusions

The genomic information on LAB and bifidobacteria has considerably expanded during the recent years, and it is to be assumed that more and more annotated genomic sequences of both species and strains will become available in the near future. The accumulated data allow us to make conclusions about the evolutionary history of these organisms, as well as to understand the factors behind their specification and ecological role.

The evolution of the present LAB has been characterized by a marked gene loss accompanied by the adaptation to nutritionally rich habitats, this adaptation being sometimes also associated with acquisition of new genes. The genetic elasticity of LAB, indicated by the genome data, is...
based on the prevalent HGT, mediated by conjugation, transduction, and transformation, as well as by the universal presence of plasmids and mobile genetic elements.

Recombinant DNA techniques can be readily applied to many LAB species and strains and are a valuable research tool, although their practical applications have been slow to materialize. However, it is to be expected that the genetic modification of LAB, combined with the more profound understanding of their genomics and provided that the safety aspects will be properly addressed, will undoubtedly help in developing the industrial use of LAB, both in their traditional and novel applications, including therapeutic use.

References


Lactic Acid Bacteria: Microbiological and Functional Aspects


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Chapter 3

Potential Applications of Probiotic, Bacteriocin-Producing Enterococci and Their Bacteriocins

Andrea Lauková

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3.1 Probiotic Potential of Enterococci

The genus *Enterococcus* as the representative of the family *Enterococcaceae*, the division *Firmicutes* (Bergey’s Manual of Systematic Bacteriology 2009), constitutes a major genus of the lactic acid bacteria (LAB). They are associated with a variety of habitats such as soil, plant, water, gastrointestinal tract (GIT) of humans and animals, rumen, and silage (Lauková et al. 1993; Devriese et al. 1995; Franz et al. 1999; Švec et al. 2001; Strompfova et al. 2003; Marciňáková et al. 2004; Simonová and Lauková 2007). They are also associated with foods (Aymerich et al. 1996; Franz et al. 1999, 2003; Čanžek Majhenič et al. 2005); most likely as a result of contamination from plant or animal sources, it has been shown that they have little value as hygiene indicators in the industrial processing of foods (Birollo et al. 2001). Varying levels of enterococci colonization have been observed in different products depending on product type, production season, and other factors (Bhardwaj et al. 2008). Enterococci also seem to play some role in numerous fermented foods. Their wide distribution in nature, when compared with other LAB, is probably explained by their persistence and their resistance to growth-inhibiting factors such as acidity, salt, drying, heat, and chemical sanitizing agents (Holzapfel et al. 2002). When considering enterococci as probiotics, their safety and/or detrimental activities are always debated. However, it is necessary to take each strain into consideration individually; for example, whether it possesses virulence determinants (hemolysin, gelatinase, *Esp* protein, etc.) as well as beneficial properties attributed to proteolytic, lipolytic, and esterolytic activities, especially in food isolates. However, there are differences concerning the virulence factors harbored by enterococci from clinical and other sources. Especially those isolated from food were found to carry a low incidence of virulence factors (Franz et al. 2001). Adhesive abilities are important for sufficient colonization of probiotic isolates and for their stability and survival in the ecosystem (Rinkinen et al. 2000; Ouwehand et al. 2001; Lauková et al. 2004a). Bacteriocin production among enterococci (mostly enterocins) has also been viewed as a potential beneficial aspect (Gálvez et al. 1989; Lauková et al. 1993; Aymerich et al. 1996; Casaus et al. 1997; Cintas et al. 1997, 1998, 2000; Floriano et al. 1998; Herranz et al. 2001; Foullié-Moreno et al. 2003a; Marekova et al. 2007; Maldonado-Barragán et al. 2009; Khan et al. 2010). Moreover, to promote safe biotechnology, the German Chemical Industry has published a list in which microorganisms have been classified into specific risk groups (Berufsgenossenschaft der Chemischen Industrie 1995). Based on this classification, Group 1 bacteria are considered not to pose any risk for human or animal health and include the majority of LAB. Group 2 bacteria are considered to have a low potential to cause infection, which is dependent on the immune status of the host, and are not generally regarded as obligatory pathogens. According to this classification, most *Enterococcus* spp. are listed under Group 2; exceptions are indicated for strains *Enterococcus faecium* and *E. durans*, which, on the basis of safe technical experience, may be considered as nonrisk strains in the sense of Group 1 organisms. The other interest has to be focused on aminogenic activity of enterococci. Latorre-Moratalla et al. (2010) tested 76 potential endogenous starter cultures for dry fermented sausages and found that aminogenic potential was strain dependent, although some species had a higher proportion of aminogenic strains than did other species. However, the enterococci tested were decarboxylase positive, producing high amounts of tyramine and considerable amounts of β-phenylethylamine. Enterococci have commonly been

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3.2 Bacteriocins Produced by Enterococci

Bacteriocins produced by enterococci are generally referred to as enterocins (Franz et al. 2007). They are ribosomally synthesized, antimicrobial peptides with activity usually directed against more or less related bacteria. Bacteriocins were classified according to the general bacteriocin classification scheme (Klaenhammer 1993; Nes et al. 1996). However, the awareness on bacteriocins produced by enterococci has enormously increased that it became necessary to reclassify the enterocin group itself. Following the general classification, enterocins were regrouped into four classes (Franz et al. 2007). Class I enterocins (these are Class I lantibiotic enterocins) are represented by cytolysins produced by some \textit{E. faecalis} strains (Booth et al. 1996). The enterocin of this class is a two-component bacteriocin consisting of two linear peptides structurally different from other linear lantibiotics such as nisin A and Z (with only one linear peptide) as well as from smaller globular peptide lantibiotics. It contains lanthionine residues, which dictates that it should be QPS probiotic (e.g., \textit{Lactobacillus casei} or \textit{Bacillus} sp.) and non-QPS probiotic (e.g., \textit{E. faecium}) (Piskoríková 2010). However, it is necessary to claim each strain in the framework of the species; to claim its antibiotic sensitivity, absence of toxins, and virulence factors; and to claim safety according to toxicological studies such as 3 pack genotoxicity studies and subchronic studies (e.g., 90 days rat study). Concerning feed, \textit{E. faecium} strains involved in the feed additive Bonvital were approved by EFSA for chicken fattening (EFSA 2010). In contrast to the enterococcal strains, enterococcal bacteriocins produced by heterologous hosts or added as cell-free, partially purified preparations have been attractive for applications in food/feed as was already experimentally proved (Giraffa et al. 1994; Lauková et al. 1999a,b, 2001; Foulquié-Moreno et al. 2003b; Ben Belgacem et al. 2010). Moreover, the British Advisory Committee on Novel Foods and Processes (1996) accepted the use of \textit{E. faecium} strain K77D as a starter culture in fermented dairy products.
be considered a two-component lantibiotic. Class II enterocins are represented by small, non-lantibiotic peptides, subdivided into Class II.1—enterocins of the pediocin family, Class II.2—enterocins synthesized without a leader peptide, and Class II.3—other linear, non-pediocin-type enterocins. Class II.1 enterocins cluster into two subgroups according to sequence similarities (Fimland et al. 2005). Subgroup 1 includes enterocin (Ent) A, the mundticins (produced by E. mundtii AT06, NFRI7393, or CUGF08), and Ent CRL5; and subgroup 2 includes Ent P (Bennik et al. 1998; Kawamoto et al. 2002; Franz et al. 2007; Feng et al. 2009) as well as Ent M, a new variant of Ent P (Mareková et al. 2007). Class II.2 includes the two-peptide bacteriocin L50 (A, B), Ent Q, and Ent C (Franz et al. 2007; Maldonado-Barragán et al. 2009). Class II.3 includes Ent B (Casaus et al. 1997; Cintas et al. 1998). Class III includes cyclic antibacterial peptides, which are represented by enterocin AS-48 produced by the clinical isolate E. faecalis S-48 (Gálvez et al. 1989; Martinez-Bueno et al. 1994). Class IV includes enterolysin A produced by E. faecalis LMG 2333 or DPC5280 or Iia (Hickey et al. 2003; Nilsen et al. 2003; Nigutová et al. 2007).

3.3 Applications of Probiotic, Bacteriocin-Producing Enterococci and Their Enterocins

Nowadays the interest in natural preservatives has increased enormously, in accordance with consumers’ demand for healthy, safe, and fresh products (Foulquié-Moreno et al. 2003b). In the framework of the FAIR Programme CT97-3078 project, some enterococci associated with food fermentations were selected in connection with their functional and safety aspects. In addition, during the Tradisausage project (Talon 2005; Lauková et al. 2011), other enterococci were selected from traditional fermented sausages. Moreover, probiotic and bacteriocin-producing enterococci were experimentally applied in different animal or waste ecosystems (Lauková et al. 1998a; Audisio et al. 2000; Pogány Simonová et al. 2009a; Szabóová et al. 2008; Strompfová et al. 2006a; Herich et al. 2010).

3.3.1 Probiotic and Bacteriocin-Producing Enterococci in Dairy Products

Enterococci have important implications in the dairy industry. They occur as nonstarter microflora in a variety of cheeses (with an impact on the development of organoleptic characteristics), in natural milk, or whey/cheese starter cultures (Giraffa 2002). There are two principal conditions where bacteriocin-producing enterococci are used in cheese manufacture: to minimize the risk of milk and cheese contamination by spoilage bacteria (Listeria spp., Clostridium sp.) in order to achieve a zero tolerance policy, and to prevent late blowing (although chemicals are still used to prevent late gas formation during cheese ripening) (De Vuyst 2004). Foulquié-Moreno et al. (2003b) reported the use of two bacteriocin-producing strains of E. faecium, RZS C5 (FAIR E-171) and DPC 1146, in cheddar cheese manufacture on a pilot scale. Enterocin production occurred at the beginning of the cheese manufacturing process and was stable during the whole ripening period. This indicated that both early and late contamination of milk or cheese by unwanted microbes can be counteracted with a stable, in situ enterocin production. The use of such a coculture is an additional safety provision beyond good manufacturing practices. Substances produced by enterococci (lactic acid and bacteriocin) are known to inhibit predominantly Listeria spp. One explanation could be their common phylogenetic development (Franz et al. 1999). Giraffa et al. (1994) presented data for the potential use of the antagonistic activity from enterococci to protect
Taleggio cheese, where *Listeria monocytogenes* may often reach dangerous concentrations, during ripening. *E. faecium* WHE 81, a multibacteriocin producer, was tested for its antimicrobial activity on *L. monocytogenes* in Munster cheese, a red smear soft cheese (Izquierdo et al. 2009). It was found that the WHE 81 strain, which naturally exists in Munster cheese, did not adversely influence the ripening process. The naturally delayed and superficial contamination of this type of cheese allowed the use of the WHE 81 strain at the beginning of the ripening as a surface culture and was found to reduce *L. monocytogenes* from a contaminated brine solution on day 7 (Izquierdo et al. 2009). When Cebreiro, a fresh or short-ripened acid curd cheese produced in the Galician mountains, was prepared with *E. faecalis* strain subsp. *liquefaciens* or *faecalis*, the cheese showed similar organoleptic characteristics to those of traditional cheeses; cheese with *E. faecalis* subsp. *liquefaciens* showed less proteolytic activity and cheese with *E. faecalis* subsp. *faecalis* had a moderate lipolytic activity (Centeno et al. 1999). Enterococcal probiotics not only exert beneficial antagonistic activity but also improve the physiological and technological properties of cheeses. To support the human immune system, *E. faecium* M74 was included in Slovak Bryndza Forte natural cheese crème.

### 3.3.1.1 Experimental Application of Enterocins in Dairy Products

Experimental application of enterocins was mainly aimed to test their antagonistic effect in different dairy products, and there have been still only limited experimental data in the literature. Enterocin 4231 is produced by a strain of non-food origin that is able to transform linoleic acid into conjugated linoleic acid (Lauková et al. 1993; Marciňáková 2006). When it was applied to skimmed milk infected with *Staphylococcus aureus* SA1 (concentration 3200 AU/ml), viable cells decreased from $10^{10}$ to $10^2$ cfu/g within 24 h. In the case of yogurt making, a significant reduction (3 log cycles) of SA1 cells was noted after 3.5 h from *Ent* addition. In yogurt, a reduction by 3.77 log$_{10}$ cfu/ml cells was also noted with the same concentration of *Ent* 4231 against *L. monocytogenes* (Lauková et al. 1999a). Moreover, *Ent* 4231 at the same concentration exhibited an antilisterial effect during the manufacture of Saint-Paulin cheese. Bacteriocin production during cheese manufacture was detected only in milk samples and curd (100 AU/ml). The pH was not influenced (Lauková et al. 2001). Antilisterial effect of *Ent* 4231 was also confirmed in traditional Bryndza Slovak cheese made from raw sheep milk with a reduction of 1 order of magnitude (Lauková and Czikková, 2001). The efficacy of enterocins in cheeses was also mentioned by Arvanitoyannis (2009) in connection with the application of the Hazard Analysis and Critical Control Point system and ISO 22000 to foods of animal origin.

### 3.3.2 Bacteriocinogenic Enterococci, Their Enterocins, and Their Applicability in Meat Products

In many countries, *Staph. aureus* is considered one of the most common pathogens causing food poisoning outbreaks, after *Salmonella*, *Clostridium perfringens*, and *Listeria* spp. (Anonymous 2001). Different meat products are produced worldwide; fermented sausages are, however, favorably consumed. Their technology involves a sequence of hurdles that appear along the ripening process. They are manufactured based on the concept of reduction of pH and/or water activity. In low-acid-fermented sausages, the absence of high acidification can be balanced by the application of additional hurdles such as bacteriocins and/or high hydrostatic pressure (HHP). Traditional and artisan-fermented meat products are manufactured mostly by small companies, farms, or local butchers. In many cases they are produced without the use of starter cultures, relying on
an endogenous microbiota to keep the traditional organoleptic qualities. Thus, the microbiota is constituted by a mixture of LAB species, including enterococci and lactobacilli as well as coagulase-negative staphylococci and yeasts (Hugas et al. 2003). Enterococci can occur in raw meats (because of their presence in the GIT of animals, which can lead to contamination of meat at the time of slaughter) (Franz et al. 2003; Simonová et al. 2006), but they are also associated with processed meats such as in traditional fermented sausages (Casaus et al. 1997; Hugas et al. 2003; Lauková et al. 2004b; Ben Belgacem et al. 2010). Enterococci are known to produce bacteriocins, and many enterococcal strains possess potential probiotic properties (Franz et al. 1999; Foulquié-Moreno et al. 2006; Ben Belgacem et al. 2010). In contrast to coagulase-negative staphylococci and lactobacilli, the biochemical activity of enterococci in a sausage matrix have not been studied in detail, but they might contribute to sausage aroma by their glycolytic, proteolytic, and lipolytic activities; metmyoglobin-reducing activity has also been described for meat enterococci (Hugas et al. 2003). 

\textit{E. faecium} RZS C13 and \textit{E. faecium} CCM 4231 were used as starter cultures for the production of a Spanish-style dry fermented sausage (Callewaert et al. 2000). The \textit{Enterococcus} strains were partially competitive during meat fermentation and strongly inhibited the growth of \textit{Listeria} spp. Moreover, the effectiveness of \textit{Ent} 4231 (produced by the earlier-mentioned \textit{E. faecium} CCM 4231) in controlling \textit{L. monocytogenes} contamination in dry fermented Slovak salami Hornád was reported (Lauková et al. 1999b). The enterocin addition resulted in the reduction of \textit{L. monocytogenes} by 1.67 log cycle in the experimental salamis compared with the control salamis immediately after addition of the bacteriocin (12,800 AU/g). Addition of \textit{Ent} 4231 in Púchov salami immediately led to a reduction in counts of \textit{L. innocua} Li1 by 0.40 log cycle from the initial count of 10^4 cfu/g. On day 2, a reduction of 1.86 log was noted compared with the control and the experimental salamis, with the highest reduction on weeks 3 and 4 (2.36 and 2.48 log, respectively) (Lauková and Turek 2004). However, in both types of the experimental salamis, bacteriocin activity itself could not be detected analytically. The antilisterial effect of enterocin was also confirmed in another type of Slovak fermented meat product, the sausage Gombasek (Lauková et al. 2003a). When \textit{Ent} M was used during Gombasek sausage manufacture (3200 AU/g) after its experimental contamination with \textit{L. innocua} Li1 strain, the initial number of Li1 was reduced by 2.7 log (comparing control and experimental samples). This reduction was prolonged up to 1 week. The pH level was not influenced as much by \textit{Ent} M as by \textit{Ent} 4231. Aymerich et al. (2000b) applied enterocins A and B (256 AU/g) produced by \textit{E. faecium} CTC 492 as semipurified food additives in different meat products. An inhibitory effect was observed on the growth of \textit{Listeria}. However, the addition of the producer strain as the sole starter culture in dry fermented sausages had no effect on the inhibition of \textit{Listeria} growth (Aymerich et al. 2000a). In contrast, the combination of both \textit{E. faecium} CTC 492 and its enterocins can prevent ropiness in sliced cooked ham (Aymerich et al. 2002). The antilisterial efficiency of \textit{Ent} CRL 35 produced by \textit{E. faecium} CRL35 against \textit{L. monocytogenes} and \textit{L. innocua} has been tested in a meat model system. The viable counts of both \textit{Listeria} species initially decreased followed by regrowth of the survivors after 1 h in the presence of the \textit{Ent} (Vignolo et al. 2000). The organoleptic properties of that meat model system were not affected. \textit{Ent} IM 4K1, produced by \textit{E. casseliflavus}, isolated from Italian sausage was used to control \textit{L. monocytogenes} contamination of Italian sausage cacciatore. Significant reduction of listerial cells was observed in cacciatore treated with the added enterocin (2400 AU/ml; Sabia et al. 2003). Ananou et al. (2005) presented the first contribution on the antistaphylococcal activity of \textit{Ent} AS-48 in a model meat sausage system. \textit{Ent AS-48} inhibits proliferation of \textit{Staph. aureus} in sausages when added at concentrations of 30 or 40 µg/g, achieving a significant reduction of 2 and 5.3 log (viable counts in cfu/g) of staphylococci with respect to the untreated control. \textit{E. faecalis} strain A-48-32, the producer of \textit{Ent} AS-48, developed well in the meat mixture, producing
sufficient amounts of Ent AS-48 to control growth of staphylococci. The best result was achieved with a bacteriocinogenic strain inoculum of 10^7 cfu/g. The addition of Ent A and B to raw sausages spiked with 3 log cfu/g of Salmonella, L. monocytogenes, and Staph. aureus showed an immediate reduction in the counts of L. monocytogenes due to the enterocins, while Salmonella was more affected by the endogenous hurdles associated with the ripening process. The application of an HHP treatment of 400 MPa at the end of ripening produced an immediate reduction in the counts of Salmonella but not in L. monocytogenes or Staph. aureus (Jofré et al. 2009). During storage of the low-acid sausages at room temperature and at 7°C, counts of Salmonella and L. monocytogenes progressively decreased in the batches, although the decrease was faster in the pressurized batches stored at room temperature. At the end of storage, Salmonella was <1 log cfu/g in the batches, but only the combination of Ent and HHP could reduce the counts of L. monocytogenes to this level. Neither the ripening process, Ent, nor the pressurization could control the levels of Staph. aureus according to Jofré et al. (2009). In general, group II bacteriocins (according to the above classification scheme), following enterocins, are the most interesting bacterial toxins for the meat industry (Nes et al. 1996; Tyopponen et al. 2003). In spite of slight regrowth of the indicator strains, strong antilisterial effect was confirmed due to different enterocins added. As no production of off-flavors was detected, it indicates the enterococci (and/or their bacteriocins) might be suitable for addition to meat as cocultures to improve food safety (Foulquié-Moreno et al. 2006). However, there are some factors limiting their activity, such as the matrix itself for their use, sensitivity of contaminant strains, bioavailability of enterocins during the food fermentation process, and their mode of action and the optimal dose. This is probably the reason why investigators always advise to select strains or bacteriocins to be used against a certain pathogen according to the target food matrix. Hermans et al. (2010) mentioned the approval of enterocin E-760 by EFSA for use in poultry meat.

3.3.3 Bacteriocinogenic Enterococci, Their Enterocins, and Their Use in Food of Non-Animal Origin

The control of food spoilage microbiota by bacteriocins has received much attention in recent years in many food matrices (Ross et al. 2002). Bacteriocins have been considered natural food preservatives, although the use of a given bacteriocin for a particular purpose generally requires specific studies. Ready-to-use vegetables are becoming increasingly popular among modern consumers. The initial microbial load of these products depends on the origin of vegetables, on agricultural practices, and on conditions of harvesting and processing (Vescovo et al. 1996). During storage, the high humidity of, for example, salad packs and the numerous cut surfaces of their components provide favorable conditions for the growth of spoilage bacteria such as pseudomonads, bacilli, or the representatives of Enterobacteriaceae (Vescovo et al. 1996; Carlin et al. 2000). Minimally processed vegetables can also be contaminated by pathogenic bacteria, this representing a health hazard (Farber and Peterkin 1991). It was also demonstrated that microbes of public health significance (e.g., Salmonella enterica serovar Typhimurium and Staph. aureus) could survive for a long time at 3°C on different types of salad (Helmy et al. 1985). Ent EJ97 produced by E. faecalis EJ97 was investigated and found to have bactericidal activity: it inhibits Bacillus maroccanus from zucchini purée (Gárcía et al. 2004). Tofu represents nonfermented soybean products. The characteristic high protein and high moisture content of tofu makes it a favorable medium for microbes. Therefore, in public health terms, tofu is classified as a potentially hazardous ready-to-eat food (Ashraf and Luczky 1990). The mean plate count of aerobic bacteria in tofu has been reported to be up to 10^7 cfu/g (Fouad and Hegeman 1993). Besides LAB, in the United States incidents involving recall of products due to contamination with L. monocytogenes have been reported in tofu (Anonymous 1992). The inhibitory effect of Ent A (P) produced by the strain E. faecium
EK13 (CCM 7419) in tofu inoculated with L. innocua was detected after 5 days (reduction of 1.17 log cycles); after 7 days a reduction of 0.92 log was noted and 0.15 log cycles after 12 days, reaching a bacteriocin activity of 204,800 AU/ml (Lauková and Mareková, 2002). Simultaneous inoculation with the bacteriocin-producing strain *E. faecium* EK13 (CCM 7419) into tofu contaminated with *L. innocua* resulted in a bacteriocin activity on days 1, 3, and 5 with a decrease on day 7 to 12,800 AU/ml and to 6,400 AU/ml on day 12. This coincided with a reduction in *L. innocua* levels by 2.37 log on day 5 after application in comparison with control tofu samples (Lauková and Mareková 2002). Effective contaminant reduction was also detected when *L. innocua* was allowed to grow for 4 days in the tofu and then bacteriocin or its producer strain CCM 7419 were applied: 2.04 log on day 3 and 2.29 log on day 5 in the case of bacteriocin addition; 2.90 log and 1.60 log on days 3 and 5 in the case of EK13 strain addition. When *Ent* was added, reduction of *L. innocua* cells was obtained immediately after *Ent* application with prolonged diminishing effect. However, the activity itself was not possible to detect. On the other hand, when the producer strain EK13 (CCM 7419) was added, the activity became possible to detect using an analytical spot test. Grande et al. (2005) reported variable interaction of *Ent* AS-48 (produced by *E. faecalis* S47) with fruit and vegetable juices with complete, partial, or negligible loss of activity. In vegetable juices, *Ent* AS-48 was very stable during 24 and 48 h of storage under refrigeration, and decay of bacteriocin activity was influenced by storage temperature. In fresh-made fruit juices, *Ent* AS-48 was stable at 4°C for 15 days and bacteriocin activity was still detectable after 30 days of storage. The efficacy of *Ent* AS-48 was also tested for biopreservation of ready-to-eat vegetable foods, such as soups and purees, against aerobic mesophilic endospore-forming bacteria (Grande et al. 2007). By adding *Ent* AS-48 (10 µg/ml), *Bacillus cereus* LWL1 was completely inhibited in tested vegetable products for up to 30 days at 6°C, 21°C, and 22°C. When a cocktail of bacteria isolated from those products was tested and found to be inhibited, a higher concentration of *Ent* AS-48 was added and all bacteria in the cocktail were inhibited. Moreover, *Ent* AS-48 acted synergistically with chemical preservatives against *S. enterica* serovar Enteritidis CECT 4300 in Russian salad (Molinos et al. 2009). For each case, the promising use of enterocins to protect foods other than those of animal origin was indicated. Of course, the up to now limited experimental use could be broadened; e.g., *E. faecium* M74 strain was used in cookies in Slovakia.

### 3.3.4 Bacteriocinogenic Enterococci, Their Enterocins, and Their Use in Silage/Feed

*E. faecalis* and *E. faecium* have been widely used as veterinary feed supplements. Since February 2004, 10 preparations (nine different strains of *E. faecium*) have been authorized as additives in feedstuffs in the EU (European Commission 2004) following the approval of Bonvital for fattening of chickens (EFSA 2010). To improve silage quality, the use of probiotic inoculants has been established (Saie 2002). Among them, mostly *Lactobacillus plantarum* or *Pediococcus*, as well as *E. faecium*, have been utilized. The selection of the best strains is essential and should be based on their safety; ability to grow over a wide range of dry matter contents, temperatures, and pH values; ability to utilize the sugars found in forages; and ability to rapidly reduce the numbers of contaminants and fungi. They also need to be stable. Jalč et al. (2009a) used *E. faecium* CCM 4231 of ruminal origin (Lauková et al. 1993), which is able to transform linoleic acid into conjugated linoleic acid (Marcinkáková 2006), for grass silage inoculation under *in vitro* laboratory conditions. The inoculant significantly increased the lactic to acetic acid ratio in silage. On day 105, the counts of CCM 4231 were 3.10 log₁₀ cfu/g from an initial 10⁹ cfu/g. When corn was inoculated by CCM 4231 strain, the counts in silage on day 105 were less than 1.0 cfu/g; however, a higher pH was noted. Significant increase in the concentration of C18:2 and
C18:3 was also noted (Jalč et al. 2009b). *E. faecium* 9296 isolated from silage was found to produce Ent (Marciňáková et al. 2008). At the end of ensiling, the strain reached counts of $10^9$–$10^{10}$ cfu/g; the counts of other LAB were also increased. However, a reduction of *Escherichia coli*, bacilli, and enterobacteria, as well as *Listeria* and molds, was noted after an aerobic stability test. A more rapid pH drop was noted, as well as a higher level of lactic acid and a decreased level of acetic and butyric acids. The effect of pH remained below 4.3 up to the end of the aerobic stability test, and an increase in dry matter was observed (Marciňáková et al. 2008). Jatkauskas et al. (2008) treated grass–legume silage with an inoculant mix containing *Lb. plantarum*, *Pediococcus*, *Lactococcus lactis*, and *E. faecium* M74 of non-silage origin. They noticed a trend wherein silage increased in dry matter and the average milk yield from cows fed the silage was improved by 1.2 kg/day. Milk composition was not affected. The pH and other silage characteristics were similar with or without the lactobacilli inoculant, with no significant differences between them.

### 3.3.5 Probiotic and Bacteriocin-Producing Enterococci and Use of their Enterocins for Farm Animals

Breeders and farmers have looked for ways to stabilize or improve the health status of farm animals. Taking into account EU regulations, alternatives to banned antimicrobial growth stimulators are desired. These alternatives are represented by probiotic bacteria with their active metabolites. For years their experimental use has been increased with the aim to allow their practical application, which is still under the stringent consideration of EU institutions such as the EFSA. However, their practical and commercial availability have been gradually improving.

#### 3.3.5.1 Ruminants

In agriculture, animal production has represented the area with the oldest tradition where probiotic implications were tested and established. The most commonly used probiotic preparations in ruminants (Svozil et al. 1986; Kmeť et al. 1990) include lactobacilli or fungi; *E. faecium* strains of non-ruminal origin have also been applied (Kmeť et al. 1993). There are several ways to utilize probiotic strains for their application in ruminants over the developmental periods: in the postnatal period as an adjunct to antibiotic treatments, for stimulation of ruminal digestion in the young. Probiotic lactobacilli reduced scouring and increased weight gain in ruminants, while *E. faecium*-containing preparations improved microbial metabolism in the rumen (Kmeť et al. 1993). *E. faecium* M74 (isolated from the stool of a breast-fed infant) was mostly applied to calves to prevent diarrhea cases in the postweaning period (Daněk et al. 1985). Moreover, ruminal bacteriocins, including those produced by *E. faecium* strains, were reported (Lauková et al. 1993; Morovský et al. 1998; Nigutová et al. 2007). Those mainly active against *Streptococcus bovis* can be used to prevent ruminal acidosis. Moreover, antidiarrheal efficacy of bacteriocin in ruminants was confirmed (Kalmokoff et al. 1996; Lauková and Czikková 1998). Another possible application of probiotics or their bacteriocins is prevention of mastitis, which occurs frequently in ruminants; this beneficial effect of probiotics was reported by Pieterse and Todorov (2010).

#### 3.3.5.2 Pigs

Enterococci are part of the endogenous microbiota in pigs (Devriese et al. 1995; Franz et al. 1999; Strompfová et al. 2006c). That is why *E. faecium*-based probiotics were first applied in pigs (Underdahl et al. 1982), specifically *E. faecium* SF68 (NCIMB10415; isolated from a human).
Gnotobiotic pigs were fed the SF68 strain and exposed to *E. coli* strains 0157:K88ac:H19 and 08:K87, K88ab:H19. This induced only mild diarrhea and none of the pigs died; they continued to eat well and gained weight. Histopathological examinations demonstrated abundant colonization of the intestinal tract by *E. faecium* SF68 (Underdahl et al. 1982). Scharek et al. (2005) later reported the beneficial effects of that strain on piglet performance, general composition of microbiota, as well as in modifying the immune response of piglets. Even *E. faecium* can also reduce the rate of carryover infections of piglets by obligate intracellular pathogens and reduce chlamydiae from 85% to 60% for control and treated animals, respectively (Pollman et al. 2005). Szabó et al. (2009) reported that treatment with SF68 improved the course of infection in weaning piglets with *S. enterica* serovar Typhimurium DT104. The probiotic treatment also appeared to result in greater production of specific antibodies against *S. typhimurium* DT104. In 2006, the company Norel & Nature incorporated in their product FECINOR *E. faecium* CECT 4515, which has a high capacity for rapid colonization of the pig intestine during the postweaning period (Díaz 2006). The *E. faecium* in FECINOR is protected by polysaccharide layers, which allow it to pass through the stomach without being affected by the low pH. It also produces enzymes in sufficient amounts, which improves feed digestibility (Díaz 2006). In piglets treated with Ent A (P)-producing *E. faecium* EK13 (CCM 7419, 10⁹ cfu/ml), significantly lower counts of *E. coli* were detected compared with the control group. The concentrations of total serum protein, calcium, hemoglobin, hematocrit, erythrocytes, and index of phagocytic activity of leukocytes were significantly higher after EK13 application and cholesterol was significantly lower. Intestinal bacteria (in the jejunum, ileum, cecum, and colon) were not influenced. The pH of the feces was lower in the group with the EK13 strain, with a higher level of lactic and propionic acids noted in the colon of the EK13 group. Daily body weight was not influenced (Strompfová et al. 2006c).

### 3.3.5.3 Poultry

Poultry breeding represents the branch of animal production where especially probiotics based on *E. faecium* strains are utilized to prevent or to reduce the risk of salmonelosis, clostridiosis, and nowadays also campylobacteriosis (Meremäe et al. 2010). Among endogenous infections in poultry, necrotic enteritis caused by clostridia has been reported. Clostridia are present in low counts in healthy poultry; however, after inadequate nutrition, dysfunction of intestinal microbiota can appear, followed by imbalance of host homeostasis. *E. faecium* CCM 4231, as mentioned earlier, is a ruminal isolate that produces bacteriocin and is able to transform linoleic acid into conjugative linoleic acid (Lauková et al. 1993; Marciňáková 2006). It is involved in the microbial preparation Inhicol, which was previously produced by IAP SAS in Košice, Slovakia. *In vitro*, clostridial strains were inhibited by the bacteriocin substance produced by the CCM 4231 strain. On the basis of this inhibition, the strain was tested in an experiment using 10-day-old chickens. A significant reduction of clostridia in wall of the crop as well as in cecal content, and a slight reduction in the cecal wall and crop content were found after 5 days administration of the CCM 4231 strain (Kmeť et al. 1992). Audisio et al. (2000) isolated *E. faecium* J96 from the crop of adult chicken (with inhibitory activity most probably due to lactic acid), which was administered orally (10⁸ cfu/ml) to chicken for both preventive and therapeutic purposes. To test the preventive effects, 30-h-old chickens received the inoculum of *E. faecium* J96 twice a day with an interval of 12 h between each dose for 3 consecutive days. To test the therapeutic effects, *E. faecium* J96 was administered to 4-day-old chickens in the same way and after challenge by *S. pullorum* M97 (10⁵ cfu/ml). The chickens that were preventively treated with J96 survived the *S. pullorum* challenge. Those that were infected on the first day of the experiment and then inoculated with J96 strain
died 4 days later. Salmonellae were isolated from their livers and spleens. Therefore, J96 can prevent S. pullorum infection in chickens but cannot act as a therapeutic agent. This was confirmed in the experiments using gnotobiotic Japanese quails. Lauková et al. (2003b) used E. faecium EK13 (CCM 7419), an Ent A (P)–producing strain, to check its preventive and therapeutic effect. The birds (3 days old) were divided into two groups. The experimental group received E. faecium CCM 7419 (10^9 cfu/ml), from the start, while the control group received placebo. Sixteen hours later, both groups were infected with S. enterica serovar Duesseldorf SA31 (10^7 cfu/ml). A reduction in the numbers of the SA31 strain, as an effect of CCM 7419, was found in the feces of samples taken at 24 and 48 h from the group with the CCM 7419 strain. Significant reductions were also detected in the numbers of SA31 cells in the cecum but not in the ileum after the birds were killed (Lauková et al. 2003b). When Ent A (P) was administered to Japanese quails following a preventive and therapeutic schedule of experimental application, a significant difference in the numbers of SA31 cells was noted in the feces of the group given the preventive treatment compared with control after 8 h, and in the group given the therapeutic treatment after 24 h. After 48 h, lower counts of SA31 were noted in both experimental groups. Numbers of SA31 were also reduced in the cecum and ileum of the group given the therapeutic treatment, but not in the group with the preventive treatment (Lauková et al. 2004c). Application of Ent A (P) before and after SA31 infection showed a protective effect in the duodenal epithelium. Damage to microscopic and submicroscopic structures of enterocytes and goblet cells was less intensive after Ent A treatment. However, prominent damage of enterocytes and their necrosis was noted in Salmonella-infected quails (Ciganková et al. 2004). In contrast to strain M97, Ent A showed stronger therapeutic effect than prophylactic effect. Vahjen et al. (2002) found in turkeys that E. faecium NCIB 10415 (SF68) stimulates other LAB as well as enterococci (which were increased 10 times in the treated group); an increase in lactate concentration was also noted. After administration of the same strain in broilers, an increase in villus height in the ileum was detected. Thus, this strain enhances weight gain and feed conversion in chickens. Horniaková and Bušta (2006) found positive effects of E. faecium 3530 from IMB52 preparation (Biomin GmbH Company, Austria) on egg weight, yolk, and white weight. E. faecium EF55 was isolated from chicken crop content (Strompfová et al. 2003). The early exposure of chickens to E. faecium EF55 at 10^9 cfu/ml for 7 consecutive days led to more rapid development of intestinal villi after infection at 8 days of age with S. enterica serovar Enteritidis compared with the untreated control. Reduced colonization of the intestinal tract by salmonellae in birds treated with E. faecium EF55 also preserved the microenvironment of the intestine from harmful effects of the pathogen (Levkut et al. 2009; Herich et al. 2010). E. faecium Al 41 is of non-chicken origin and is known to produce Ent M and possesses probiotic properties (Mareková et al. 2007). This strain was tested in a model experiment using laying hens (Lauková et al. 2009a). The highest counts of the AL41 strain was reached after 2 weeks of its application (up to 10^3 cfu/g) and at the end of experiment the total enterococcal counts were not diminished. Significantly higher phagocytic activity was found in birds with AL41 strain than in the control birds. Concerning campylobacteriosis, Svetoch and Stern (2010) reported the possible use of bacteriocins to control Campylobacter spp. in poultry.

### 3.3.5.4 Rabbits

In rabbits, the majority of digestive events occur in the cecum, where a wide variety of microbial population resides. Most studies deal with the strictly anaerobic and facultative bacteria (Canganella et al. 1992); their counts depend on the age of the rabbit. Because during the post-weaning period there occurred many health problems in rabbit husbandry: there is a potential
for probiotic application. The commonly used probiotic lactobacilli are not relevant for use in rabbits because they are only minor members of the microbiota. However, enterococci are present in the rabbit microbiota in substantial numbers (Simonová et al. 2005). Although up to now Bacillus-based probiotics are used in rabbitries (Kristas et al. 2008), successful results have recently been published on the application of bacteriocin-producing probiotic enterococci and their enterocins to limit unsuitable microbes in the rabbit digestive tract, to stimulate immune activity, to increase weight gain, and reduce Eimeria spp. oocysts (Chrastinová et al. 2007, 2010; Szabóová et al. 2008; Pogány Simonová et al. 2009b; Lauková et al. 2009b). When E. faecium EF2019 (CCM 7420, isolated from a rabbit) was administered to rabbits via drinking water, an increase in body weight of animals was achieved. A reduction of fecal E. coli, coagulase-positive staphylococci (CPS), Staph. aureus, and Clostridium-like bacteria was also achieved after administration of Ent 7420 (Pogány Simonová et al. 2009a). In the cecum, a significant reduction of CPS was noted in both experimental groups of rabbits—those administered with CCM 7420 and those with Ent 7420 for 3 weeks. In rabbits fed CCM 7420, the lowest activity of glutathione peroxidase was measured, which indicates that administration of this strain did not evoke oxidative stress. Moreover, after 21 days of Ent 7420 application, a reduction of Eimeria spp. oocysts was recorded (Pogány Simonová et al. 2009a), which is an important result, taking into account that from the year 2012 the use of coccidiostats will be banned in the EU. The in vivo reduction of Eimeria oocysts is in accordance with our in vitro studies in which Eimeria spp. oocysts were also reduced using probiotic enterococci or Lb. fermentum AD1 strain (Strompfová et al. 2010). When freeze-dried CCM 7420 was administered to rabbits and was compared with a fresh culture, the highest fecal counts of CCM 7420 were measured in samples administered the fresh culture (Pogány Simonová et al. 2008). However, a high energy value of rabbit meat was achieved with both fresh and freeze-dried CCM 7420 (Pogány Simonová et al. 2008). Decrease of Staph. aureus and Eimeria spp. oocysts was noted in both cases, but higher levels of biochemical parameters were found in the serum of the fresh culture group of CCM 7420. E. faecium AL41 is a strain of non-rabbit origin, producing Ent M (Mareková et al. 2007). Although it only slightly colonized the rabbit’s GIT, a reduction of CPS and pseudomonads was noted in the cecum. However, higher phagocytic activity was measured in rabbits administered E. faecium AL41 than in control animals, and higher in those administered with strain than with Ent M administration (Lauková et al. 2008a, 2009b). E. faecium CCM 4231 is also a strain of non-rabbit origin and in spite of its slight colonization in rabbits (up to 10^3 cfu/g), it was found to reduce CPS and Clostridium-like bacteria (Szabóová et al. 2008). Also a reduction of Eimeria spp. oocysts was noted, from 117 oocysts per gram (OPG) to 83 OPG, and animals achieved higher weight gain and lower mortality. Moreover, improved amino acid composition in meat and preserved meat quality and nutritional value were observed after CCM 4231 supplementation (Pogány Simonová et al. 2009b). These studies have repeatedly shown the beneficial effects of probiotic enterococci on the rabbits’ microbiota and health, independent of the origin of the probiotic E. faecium strain used. The most important effect noted was the reduction of Eimeria spp. oocysts.

3.3.5.5 Dogs

Some probiotic products are commercially available for use in dogs. However, probiotic bacteria contained in those products are of non-canine origin, for example, Probican paste with E. faecium M74 (Medipharm s r.o., Czech Republic), Enteroferm® containing E. faecium NCIB 10415 (SF68; Vahjen and Männer 2003), and FortiFlora (Purina, containing live strains of
E. faecium SF68 and antioxidant vitamins). Because pet owners desire stable health conditions for their dogs, they are interested in probiotic bacteria. Among non-Enterococcus probiotic bacteria, Biourge et al. (1998) tested Bacillus CIP 5832, which is commercially available in its sporulated form as a powder (Paciflor; Prodeza, Vannes, France) at the concentration of 10^{10} cfu/g dry dog food. The strain was found to survive and germinate in the GIT of healthy dogs, and has previously been shown to have beneficial effects as a feed additive on the breeding performances of rabbits, pigs, chickens, turkeys, ducks, calves, and horses (Lestrader 1995). Lb. rhamnosus GG is a commercially used probiotic strain, isolated from a human, and has been tested as a potential probiotic agent in healthy dogs. However, this strain was found to exhibit variable fecal colonization in healthy dogs (Weese and Anderson 2002; Strompfová et al. 2006b). Strompfová et al. (2006a, 2007) administered Lb. fermentum strain AD1 (CCM 7421, 10^9 cfu/g), of canine origin, to healthy dogs as well as to dogs suffering from gastrointestinal diseases. In both cases, the strain was administered for 7 days. After administration, numbers of fecal lactobacilli increased significantly in the feces of healthy dogs. Moreover, after 6 months the strain was still detected in dog feces in amounts of 10^3–10^5 cfu/g. A significant increase in total proteins and lipids, and a significant reduction in glucose levels in the serum of dogs were also noted. This means that Lb. fermentum survived transit through the canine GIT and populated the colon and probably improved absorption of some nutrients. In diseased dogs, a significant increase of LAB was also noted after CCM 7421 administration and E. coli counts were reduced in the majority of dogs. A significant decrease of alanine aminotransferase was detected in dogs with acute GIT diseases. On the other hand, an increase of total protein in dogs with hypoproteinemia was noted as well as the regulation of cholesterol level. Watery feces returned to normal consistency in a short time in most dogs. Because the adhesive ability of lactobacilli is mostly host dependent (Rinkinen at al. 2000; Ouwehand et al. 2001), in contrast to enterococci under in vitro conditions (Lauková et al. 2004a), enterococci would probably be more suitable probiotics for dogs. Benyacoub et al. (2003) reported that supplementation of dog food with E. faecium SF68 stimulates the immune functions in young dogs. Marciňáková et al. (2006) isolated and characterized the bacteriocin-producing and probiotic strain E. faecium EE3 from canine feed. This strain was administered to healthy dogs (10^9 cfu/g) for 7 days. Consumption of EE3 was not associated with any adverse clinical effects. The average concentration of EE3 strain in dog feces was 4.85 ± 2.43 log_{10} cfu/g on day 7, with prolonged survival of up to almost 3 months after cessation of strain feeding. There was also a reduction of staphylococci and Pseudomonas-like bacteria. Abnormal cholesterol levels were brought to physiological levels (Marciňáková et al. 2006). Moreover, Cvrčková (2009) and Lauková et al. (2010) tested in dogs the effect of E. faecium strains of non-canine origin, both bacteriocin producing and probiotic. They were applied for 7 or 14 days at a concentration of 10^9 cfu/g, and were found to control not only the microbial profile but also phagocytic activity. E. faecium EK13 (CCM 7419) was isolated from animal waste (Lauková et al. 1998b), and it produces enterocin A (P) (Mareková et al. 2003). On the other hand, E. faecium CCM 4231 is of rumen origin and produces enterocin 4231 (Lauková et al. 1993, strain deposited at CCM). The average counts of CCM 7419 on day 7 in healthy dogs reached log_{10} 5.6 cfu/g, persisting up to 3 months at a level of 2.98 log_{10} cfu/g. Counts of 10^5 cfu/g were detected in healthy dogs after colonization by CCM 4231 strain; 7 and 14 days after cessation of its administration, 10^3 cfu/g colonies were still detected. A decrease in the numbers of staphylococci, Clostridium-like bacteria, pseudomonads, and aeromonads was noticed in fecal samples of dogs receiving both strains CCM 7419 and CCM 4231. Moreover, phagocytic activity was either increased or not influenced. It seems that the canine GIT is receptive for the colonization of probiotic strains isolated from both canine and non-canine origins.
3.3.5.6 Other Animals

Some preparations are commercially available for cats, for example, Fortiflora containing *E. faecium* SF68 and antioxidant vitamins, as well as Feline Dophilus containing *Lb. acidophilus* and *E. faecium* SF68. They are, however, not isolates from cats.

For horse breeders a commercially available preparation is Probios®, containing a mixture of probiotic strains of *Lb. acidophilus*, *Lb. casei*, *Lb. plantarum*, and *E. faecium*, which are not of equine origin. Raga et al. (2008) isolated vaginal LAB from mares and found *Enterococcus* sp. with promising features for use as equine probiotics. Similarly, Lauková et al. (2008b) reported the potential probiotic applications of enterococci isolated from a healthy horse; those strains are also bacteriocin producing.

3.3.6 Probiotic Enterococci and Aquaculture

Aquaculture is an economically important activity in many countries. Prevention and control of diseases in aquaculture have led to a substantial increase in the use of veterinary medicines. However, the utilization of antibiotics as a preventive measure has been questioned, given the extensive documentation of the evolution of antimicrobial resistance among pathogenic bacteria (Balcazar 2003). There is an increasing interest within the industry at present in the control or elimination of antimicrobial use. Therefore, an alternative method that is gaining importance within the aquaculture industry is the use of probiotic bacteria to control pathogens (Sahu et al. 2008). However, there are still limited data on the use of probiotic bacteria in aquaculture. Thus, this remains an open research area. Among LAB, the probiotic strains of *E. faecium* can be considered for use in aquaculture. Swain et al. (2009) reported that *E. faecium* MC13 isolated from fish intestine effectively inhibited shrimp pathogenic bacteria such as *Vibrio harveyi* or *V. parahaemolyticus*. When MC13 was added in a tank containing 500 postlarval shrimp (10^7 cfu/ml), the mortality was 16% compared with the control (40%) and the other tested probiotic strain, *Lactococcus garviae* B49. When feed of postlarval *Penaeus monodon* was enriched with *E. faecium* MC13, the mortality of shrimp was also reduced (Swain et al. 2009). When *E. faecium* SF68 (a probiotic strain of human origin) was used in feed (10^6 cfu/ml) for European eels (*Anguilla anguilla* L.), the intestine of the eel was colonized on day 4 postinoculation at 10^5 cfu/g; on day 14 the strain constituted 73% of the intestinal microbiota (Chang and Liu 2002). They also observed that survival rates of eels fed with *E. faecium* SF68 supplement were significantly higher than those of control eels or those fed with *Bacillus toyoi* supplement. These results suggest that the bacterial probiotics can be used to control pathogenic bacteria in aquaculture.

Concerning animals, it can be concluded that probiotic, bacteriocin-producing strains and their enterocins showed mostly antimicrobial effect to control unsuitable microbiota members and stimulate the organism’s immune response by increasing phagocytic activity. They did not evoke oxidative stress. They beneficially, or at least did not negatively, influenced daily weight gain and biochemical parameters in serum.

3.3.7 Enterococci, Their Enterocins Used in Animal Waste

Agricultural production has a marked effect on the quality of the environment. The problems of hygiene and ecological hazards associated with waste storage are important in the European context. Traditional aerobic and/or anaerobic treatments of slurry in thermophilic and mesophilic zones are designed to stabilize the treated matter, but it is insufficient for complete decontamination
of the excrements. Therefore, posttreatment methods are being sought to achieve higher effectiveness in bacterial decontamination of stabilized waste and/or slurry. Concerning the environment, enterococci are always considered to be indicators of fecal contamination in water and they are also present in animal waste (Godfree et al. 1997; Lauková et al. 1998b). When enterocins 4231, V24, and EC24 were used for the treatment of pig slurry infected with different pathogenic bacteria, Ent V24 produced by \textit{E. faecalis} inhibited the growth of \textit{Enterobacter cloacae} as well as \textit{Pseudomonas} spp. (Lauková et al. 2002). Also in dung of cattle given water experimentally contaminated with \textit{L. monocytogenes} Ohio and \textit{Yersinia enterocolitica}, substantial reductions in microbial counts were already noted after 1 h of \textit{Ent} V24 addition, whereas \textit{Ent} 4231 addition led to reduction of \textit{L. monocytogenes} (Lauková et al. 1998a; Lauková et al. 2000). There is a need to continue with these types of treatments and to discuss about “environmental” probiotics.

### 3.3.8 Probiotic Enterococci in Man

Lyophilized \textit{E. faecium} M74 was administered to adults at a daily oral dose of $5 \times 10^9$ cfu/ml for 6 weeks. The bacterium temporarily colonized the host intestine, and its excretion with stool persisted for 5 weeks after the last dose. The strain showed hypcholesterolemic and immunostimulatory effects (Mikeš et al. 1995; Ebringer et al. 1995; Hlivák et al. 2005).

### 3.4 Conclusions

Enterococci have been shown to have a positive influence on the health of a variety of hosts. Combined with their resilience, it makes them excellent probiotic candidates. The issue of antibiotic resistance transfer, however, remains and should be judged on a strain-by-strain basis as is in any case common for probiotics.

### References


Lactic Acid Bacteria: Microbiological and Functional Aspects


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Chapter 4

Genus Lactococcus

Atte von Wright

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4.1 The Taxonomic Unit Defined

The genus Lactococcus (formerly “Group N Streptococci”) comprises seven species, Lactococcus lactis (including the subspecies cremoris, lactis, and hordiae), L. garvieae, L. piscium, L. plantarum, and L. raffinolactis (Batt 2000; Schleifer et al. 1985; Doménech et al. 1993; Williams et al. 1997), L. chungan-gensis (Cho et al. 2008), and the quite recently characterized L. fujizensis (Cai 2010). Species identification is based on physiological, chemotaxonomic, and molecular biological criteria. Morphologically,
### Table 4.1 Physiological Characteristics Differentiating *Lactococcus* Species

<table>
<thead>
<tr>
<th></th>
<th><em>L. lactis</em></th>
<th><em>L. garvieae</em>&lt;sup&gt;c&lt;/sup&gt;</th>
<th><em>L. piscium</em></th>
<th><em>L. plantarum</em></th>
<th><em>L. raffinolactis</em></th>
<th><em>L. chungangensis</em></th>
<th><em>L. fujensis</em></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Growth at 4°C</strong></td>
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<td></td>
<td>+</td>
<td></td>
<td>–</td>
<td>+</td>
<td>ND</td>
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<tr>
<td><strong>Growth at 40°C</strong></td>
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<td>+</td>
<td></td>
<td>–</td>
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<td>–</td>
<td>+</td>
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<tr>
<td><strong>Growth at 4% NaCl</strong></td>
<td>+&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Occasionally</td>
<td>ND</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Lactose fermentation</strong></td>
<td>+&lt;sup&gt;b&lt;/sup&gt;</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
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<tr>
<td><strong>Mannitol fermentation</strong></td>
<td>–</td>
<td>Occasionally</td>
<td>+</td>
<td>+</td>
<td>ND</td>
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<tr>
<td><strong>Raffinose fermentation</strong></td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>ND</td>
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<tr>
<td><strong>Starch fermentation</strong></td>
<td>±</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>±</td>
<td>ND</td>
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<tr>
<td><strong>Hydrolysis of aesculin</strong></td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>ND</td>
</tr>
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</table>

**Note:** ND, not determined.

<sup>a</sup> Except for subspecies *cremoris* and *hordniae*.

<sup>b</sup> Except for subspecies *hordniae*.

<sup>c</sup> Tolerates 3% NaCl.
lactococci are gram-positive cocci of 0.5–1.5 µm in size, forming short chains. They are mesophilic, ferment hexoses homfermentatively producing L (+) lactic acid, and have complex growth requirements. Some physiological characteristics differentiating the species are listed in Table 4.1.

In practice, it is often difficult to distinguish between *L. lactis* and *L. garvieae*. Pyrrolidinylarylamidase activity has been considered specific for the latter species, but *L. lactis* strains having this activity are also frequently met (Elliot et al. 1991). Thus molecular biological identification is usually necessary to obtain a reliable identification.

### 4.2 Typical Ecological Niches

Lactococci have been isolated from vegetable materials (Cai et al. 2010), milk, or other animal sources, including human gut (Kubota et al. 2010). *L. chungangensis* was originally isolated from activated sludge foam (Cho et al. 2008).

*L. lactis*, the species most commonly found in raw milk, is thought to arise as a contamination from forage. *L. garvieae* was originally isolated from a mastitis case (Collins et al. 1983) but is better known, together with *L. piscium*, as a fish pathogen. *L. garvieae* typically occurs in warm water species while *L. piscium* occurs at temperatures below 15°C (Venderell et al. 2006; Williams et al. 1990). *L. garvieae* can also frequently be isolated from dairy foods (Fortina et al. 2003), and recent findings suggest that the fish isolates of *L. garvieae* are lactose negative, while dairy isolates ferment lactose (Fortina et al. 2009).

### 4.3 Dairy Lactococci

*L. lactis* subsp. *lactis* and *cremoris* are the lactococci traditionally used in dairy applications. The main difference between the subspecies is the salt tolerance and ability to hydrolyze arginine, both typical for subsp. *lactis* but absent in *cremoris*. The diacetyl-producing variants of *L. lactis* subsp. *lactis* are often referred as biovar. *diacetylactis* (Batt 2000).

#### 4.3.1 Starter Use

Dairy lactococci, often together with *Leuconostoc mesenteroides* subsp. *cremoris*, are essential for the mesophilic dairy starters used both for the production of fermented milks and cheeses (Stanley 1998; Courtney 2000). Mesophilic starters are often further classified according to the species composition, the starters containing a single *L. lactis* strain being called 0-starters, while D, L, and DL starters also contain aroma producers, either *L. lactis* subsp. *lactis* biovar. *diacetylactis*, *Lc. mesenteroides*, or both, respectively.

#### 4.3.2 Metabolic Characteristics

While dairy lactococci share the basic physiological features of other lactic acid bacteria (LAB), the pathways of their sugar fermentations, proteolytic systems, and genetic basis have been extensively studied owing to their importance to the dairy industry. The availability of genomic and proteomic data and techniques such as in vivo nuclear magnetic resonance spectroscopy are also making the metabolomic approaches feasible. In the following paragraphs, some of the key metabolic features of dairy lactococci are briefly summarized.
4.3.2.1 Carbohydrate Metabolism

4.3.2.1.1 Sugar Transport

The central hexose transport system in dairy lactococci is the phosphoenolpyruvate phosphotransferase system (PEP-PTS; reviewed by Postma et al. 1993; Deutscher et al. 2006). In PEP-PTS the uptake of sugars is coupled to their phosphorylation, phosphoenolpyruvate (PEP) acting as the donor of the phosphate group via several steps. PEP first phosphorylates the so-called enzyme I (EI), which subsequently transfers the phosphate group to heat-stable protein (HPr-P). From HPr-P the phosphate is transferred via enzymes EIIA and EIIBC/EIICD to the carbohydrate to be transferred. EI and HPr are nonspecific cytoplasmic proteins, while EII enzymes are sugar specific and linked to the membrane by hydrophobic domains C or D. Glucose transport in dairy lactococci occurs either via the mannose-PTS (the major route) or in some cases by a specific glucose-PTS (Thompson 1987; Thompson and Saier 1981). Regarding the applications, the most important sugar is lactose, which is mainly transported by a specific PTS (de Vos et al. 1990).

In comparison to the extensive information available on hexose transport, the details of pentose transport in lactococci have not received much attention. Apparently they are transported mainly by specific permeases or symporters, as in other bacteria (Erlandson et al. 2000).

4.3.2.1.2 Sugar Fermentation

The hexose fermentation of lactococci occurs homofermentatively. While the fermentation of the 6-phosphorylated glucose produced by the PTS system proceeds in a straightforward manner via the glycolytic pathway, the fermentation of phosphorylated lactose requires the action of phospho-β-galactosidase, which splits the molecule to glucose and galactose-6P. Thereafter these two moieties have different fates. Glucose will be phosphorylated and enter glycolysis, while galactose-6P is isomerized to tagatose-6-P. After a further phosphorylation tagatose-1,6P is formed, and subsequently split to two triose-3P molecules. These will then be metabolized to pyruvate and finally to lactic acid via the normal glycolytic route. The details of hexose metabolism in \textit{L. lactis} have been reviewed by Neves et al. (2005).

The pentoses have to be processed by different isomerases and epimerases to xylulose-5P, from which the heterolactic phosphoketolase pathway proceeds to yield lactic acid and ethanol/acetaldehyde (Kandler 1983).

4.3.2.2 Alternative Pyruvate Metabolism, Diacetyl Formation

While most of the pyruvate formed in sugar metabolism is reduced to lactic acid, in certain circumstances pyruvate is also directed to other metabolic pathways leading to a variety of end products (diacetyl, acetoin, butanediol, acetate, formate, ethanol, CO$_2$). The detailed pathways are outlined in Chapter 1.

Industrially the most important of these secondary pathways is the formation of diacetyl, the important aroma component of dairy products (“butter flavor”). If hexoses are the only sources of pyruvate, the formation of diacetyl is low. In dairy processes, the citrate, present in milk at 8–9 mM quantities, provides the main source of pyruvate for diacetyl formation. The aroma-producing lactococci (\textit{L. lactis} subsp. \textit{lactis} biovar \textit{diacetylactis}) possess citrate permease and lyase enzymes. The lyase splits citrate to acetate and oxaloacetate. Oxaloacetate is then decarboxylated to CO$_2$ and pyruvate (Hugenholz 1993). The activity of the citrate transport in lactococci is controlled by external pH (Garcia-Quintans 1998).
4.3.2.3 Proteolytic System and Peptide Utilization

Lactococci are fastidious organisms and strain dependently auxotrophic for many amino acids, the amount of which in milk is limited. The dairy lactococci have an elaborate proteolytic system coupled with peptide transport, allowing them to obtain the necessary amino acids from the breakdown products of milk proteins. The system consists of a cell-envelope protease (CEP), peptide transport systems, and intracellular peptidases (for reviews, see Savijoki et al. 2006; Doeven et al. 2005). While this machinery or some of its components also operate in other LAB, it is by far best characterized in dairy lactococci.

The CEP of *L. lactis*, PrtP, was the first cloned and characterized LAB CEP (Kok et al. 1988). Subsequently the functional domains of CEPs have been further elucidated (Siezen 1999; see also Chapter 1). The enzyme is a typical serine protease of subtilisin type, anchored to the cell wall by the C-terminus, and cleaves casein to oligopeptides of variable sizes. The generated peptides can be transported inside the cell by several peptide transport systems. The most important of them are the oligopeptide transport systems (Opp and Dpp), which both represent ATP-binding cassette transporters. The Opp system was first identified by cloning a chromosomal fragment enabling a spontaneous *L. lactis* mutant deficient in peptide transport to grow in milk (Tynkkynen et al. 1989). Subsequently, the fragment was shown to contain an operon coding for the oligopeptide binding protein (OppA), two membrane proteins (Opp B and OppC), and two ATP-binding proteins (OppD and OppF) (Tynkkynen et al. 1993).

Opp is responsible for the transport of peptides ranging in size up to 18 amino acids. Smaller peptides are handled by di- and tripeptide transporters, such as DtpT and Dpp, Dpp being able to a degree complement the absence of the functional Opp in some lactococcal strains (Hagting et al. 1994; Foucaud et al. 1995).

Once inside, the peptides are broken down by a large number of different peptidases representing metallopeptidases, cysteine peptidases, and serine peptidases. Both endopeptidases, aminopeptidases, di- and tripeptidases, and several proline peptidases have been characterized (Christensen et al. 1999; Liu et al. 2010).

4.3.3 Genetics

Natural gene transfer mechanisms well characterized in lactococci include transduction, observed already in early 1960s (Sandine et al. 1962; Allen et al. 1963), and conjugation (for a review, see Fitzgerald and Gasson 1988). The so-called sex factors associated with a high frequency of conjugation have also been characterized (Gasson et al. 1995).

Although natural transformation of lactococci have been reported occasionally (Møller-Madsen and Jensen 1962), and complete competence operons (competence meaning the capacity to intake foreign DNA through the cell wall) has been detected in sequenced *L. lactis* genomes (Wydau et al. 2006), natural transformation is not a generally applied gene transfer method in *L. lactis*. The *in vitro* transformation methods include protoplast transformation (Kondo and MacKay 1984; von Wright et al. 1985) and electroporation (Holo and Nes 1987), the latter being the current standard technique.

Lactococci typically contain a large number of plasmids of variable sizes, many of them associated with important functions relevant for their applications in dairy products (see below). Accordingly, the study of lactococcal plasmids dominated the early research, but the rapidly expanding sequence data will undoubtedly shift the focus of the research on the actual chromosomal genetics of these organisms.
4.3.3.1 Lactococcal Plasmid Biology

4.3.3.1.1 Metabolic Plasmids

Plasmids coding for important technological properties have naturally attracted much attention since their discovery in the lactococci (McKay et al. 1972; Cords et al. 1974). In these bacteria, lactose fermentation and proteinase activities are almost invariably associated with relatively large (from 17 kbp to more than 50 kbp) plasmids (McKay 1983). The linkage of citrate permease gene in diacetyl-producing *Lc. lactis* to small (approx. 8.7 kbp) plasmids was also detected relatively early (Kempler and McKay 1981).

Mucoidness, or the ability to produce extracellular polysaccharides, is a property of some lactococcal strains that have traditionally been used to give body and texture to certain types of Scandinavian fermented milks (Macura and Townsley 1984). Several lactococcal plasmids ranging in size between 27 and 47 kbp associated with mucoid phenotype have been identified (Vedamuthu and Neville 1986; von Wright and Tynkkynen 1987; Neve et al. 1988). Genetic analysis of one of the plasmids, pNZ4000, has indicated the involvement of at least 14 genes in exopolysaccharide production (van Kranenburg et al. 1997). The complete sequence of this 42.2 kbp plasmid has been elucidated (van Kranenburg et al. 2000).

4.3.3.1.2 Bacteriocin Production Plasmids

Bacteriocins are bacterial proteins or peptides that inhibit the growth of other bacterial strains or species. Bacteriocins are classified into three groups (Nes et al. 1996): class I containing modified amino acids such as lanthionine and β-methyllanthionine, small heat-stable nonlantibiotics (class II), and large heat-labile bacteriocins (class III). Several plasmid-encoded bacteriocins have been characterized in dairy lactococci. However, nisin, the only lactococcal bacteriocin with industrial applications, is not associated with plasmids but with special chromosomally located transposons (see Section 2.5.1).

Lacticins represent plasmid-encoded class I bacteriocins. Lacticin 481 coded by a six-gene operon located on a 70 kbp plasmid in *Lc. lactis* ADRIA 85LO30 (Rincé et al. 1997) is a typical example. Another lactococcal plasmid-encoded bacteriocin belonging to the class I bacteriocins is the two-component lantibiotic lacticin 3157 encoded by a 60 kb plasmid, which has been completely sequenced (Dougherty et al. 1998).

Lactococcins belonging to the class II bacteriocins are mainly active against other lactococci. An illustrative example of a plasmid coding for multiple bacteriocins is p9B4-6 from *Lc. lactis* subsp. cremoris 9B4 (Neve et al. 1988). In this plasmid, one bacteriocin locus consisting of two genes jointly produce the so-called lactococcin M (low antagonistic activity) while the other region coded for two independent highly active lactococcins (A and B) (van Belkum 1992).

4.3.3.1.3 Plasmids and Phage Defense Mechanisms

Plasmids associated with increased resistance against bacteriophages are common, especially among the lactococci. Three basic phage resistance mechanisms with different subdivisions are known: inhibition of phage adsorption, restriction/modification (R/M) systems, and abortive infection (Abi) or intracellular inhibition of phage development. Tens of plasmids coding for these mechanisms are known (Hill 1993; Daly et al. 1996; Chopin et al. 2005). Even a cursory attempt to describe them in this context is obviously out of the scope of this section.
A well-characterized 65 kbp conjugative plasmid, pNP40, from *Lc. lactis* ssp. *lactis* biovar *diacetylactis* DRC3 is an illustrative example of lactococcal plasmids associated with multiple functions. pNP40 confers resistance against the bacteriocin nisin and also protects the strain from the attack of the lytic bacteriophage c2 (McKay and Baldwin 1984). Subsequently, the plasmid has been shown to contain genes for at least two Abi systems (AbiE and AbiF) and a restriction–modification system (LlaJI), and for a mechanism blocking phage DNA injection (Garvey et al. 1995, 1996; O’Driscoll 2004). The fact that this plasmid also codes for cadmium resistance, which can be used as a selective marker, further emphasizes its potential usefulness in engineering dairy starter strains for enhanced phage resistance (Trotter et al. 2001). Among the other genes characterized in this plasmid are a homolog of *recA* (a gene central in DNA recombination and repair) as well as a gene sharing homology with *umuC* (a gene involved in the so-called SOS response to DNA damage) (Garvey et al. 1997). These findings suggest a possible role for genes active in DNA recombination and repair in some of the Abi mechanisms.

4.3.3.1.4 Antibiotic Resistance Plasmids

Although lactococci are generally sensitive to most antibiotics in clinical use, occasional antibiotic resistances have been observed, based on both chromosomally located and plasmid-linked genes. From the safety point of view, the possibility of the horizontal transfer of these resistances is of particular interest.

A 30 kbp completely sequenced theta-replicating plasmid pK214 coding resistance for streptomycin, tetracycline, and chloramphenicol, with the resistance determinants apparently related to corresponding genes in *Staphylococcus*, *Listeria*, and *Enterococcus*, has been detected in a lactococcal strain isolated from soft cheese. The plasmid also contains five insertion (IS) elements, three of which apparently originating from *Enterococcus faecium* (Teuber et al. 1999).

Plasmids carrying the Tn916 transposon and the associated tetracycline resistance gene *tet(M)* have been detected in lactococci isolated from raw milk (Belén Flórez et al. 2008), and this resistance could be conjugatively transferred to *Lactococcus* and *Enterococcus* recipients.

Another tetracycline resistance gene, *tet(S)*, located on a plasmid of an *L. lactis* strain of fish origin, has been successfully electroporated into *L. garvieae*, which was subsequently used as donor to conjugate the plasmid into *Listeria monocytogenes* (Guglielmetti et al. 2009).

4.3.3.2 Genetics of Nisin Biosynthesis

Nisin is a broad-spectrum lantibiotic bacteriocin produced by *L. lactis*, and the only bacteriocin that has been accepted as a food additive (E234) to control contaminating gram-positive bacteria (Courtney 2000; see also Chapter 15).

The genes for nisin biosynthesis and immunity as well as for sucrose utilization are known to reside on a 70 kbp conjugative transposon. This block is flanked by direct repeats of TTTTTG, representing probably a duplication of the target sequence due to transposition. However, no inverted repeats flanking the nisin–sucrose gene block have been identified (Rauch and de Vos 1992). The nisin–sucrose transposons vary slightly from strain to strain, which has resulted in different designations (Tn5301, Tn5276, Tn5307, etc.). The conjugative nature of the nisin–sucrose transposons allow for their introduction even to heterologous hosts, such as dairy enterococci (Broadbent et al. 1995).
4.3.3.3 Sequenced Lactococcal Genomes

To date, four complete genome sequences of dairy lactococci (\textit{L. lactis} subsp. \textit{lactis} IL1403, \textit{L. lactis} subsp. \textit{cremoris} SK11, \textit{L. lactis} subsp. \textit{cremoris} MG1363, and \textit{L. lactis} subsp. \textit{lactis} KF147) have been published (see Chapter 2). Characteristic to the lactococcal genomes is a large number of IS elements and transposons (Bolotin et al. 2001; Makarova et al. 2006), indicating a high degree of plasticity. In the strain KF147, isolated from plant material, several genes coding the degradation of xylan, arabinan, glucans, and fructans and for uptake and conversion of cell wall degradation products were detected, indicating adaptation to the peculiar ecological niche (Siezen et al. 2010).

4.3.3.4 Genetic Modification of Lactococci

Since both \textit{in vitro} transformation methods and a range of cloning vectors have been available since early 1980s, recombinant DNA techniques have been a standard research tool in the molecular biological study of lactococci. The first cloning vectors were based on cryptic lactococcal plasmids with added antibiotic resistance genes as selection markers (de Vos and Simons 1994). Subsequently a variety of expression and integration vectors have been developed (see Mills et al. 2006 for a review). Particularly the nisin-controlled expression system, based on the ability of the extracellular nisin to act as a transcriptional activation of its own synthesis, has been a useful tool for the expression of heterologous proteins in \textit{L. lactis} (Kleerebezem et al. 1997).

While several food-grade selection systems based on bacteriocin resistance, carbohydrate metabolism, cadmium resistance, and others, have also been proposed (Mills et al. 2006), genetically modified lactococcal strains have not thus far been used in food applications. Instead, their use for the production of therapeutic proteins and oral vaccines could be a promising option. In their review Bahey-El-Din et al. (2010) list tens of bacterial and viral antigens, immunomodulatory and therapeutic proteins that have been expressed in \textit{L. lactis}. An \textit{L. lactis} strain expressing human interleukin-10 has actually been used in a human phase I trial in patients with Crohn’s disease (Braat et al. 2006).

4.4 Potential Uses of Lactococci Other than \textit{L. lactis}

Thus far \textit{Lactococcus lactis} ssp. \textit{lactis} and \textit{cremoris} are the only lactococci used as dairy starters. The potential of a few strains of \textit{L. lactis} ssp. \textit{bordiariae}, \textit{L. raffinolactis}, \textit{L. garvieae}, \textit{L. piscium}, and \textit{L. plantarum} in dairy applications has been assessed by Holler and Steele (1995). The strains were characterized for phage resistance, lactose fermentation, and growth in milk supplemented with glucose and casein hydrolysate. The most promising were the \textit{L. raffinolactis} strains; however, even they lacked proteinase activity, and the activity was not expressed even after the introduction of the proteinase-associated plasmid from \textit{L. lactis} ssp. \textit{cremoris}. \textit{L. garvieae} has been proposed for starter culture preparations, due to its prevalence in artisanal Italian cheeses and its potential contribution to the typical sensory characteristics of traditional cheeses (Fortina et al. 2007). A possible field of application for novel lactococci could be vegetable and silage fermentations, considering the common association of lactococci and plant materials (Cai et al. 2010).
4.5 Safety Aspects

4.5.1 Dairy Lactococci

*L. lactis* ssp. *lactis* and *L. lactis* ssp. *cremoris* have been consumed in large quantities in dairy products for thousands of years, and have a remarkably well established history of safe use. However, cases of endocarditis (Halldorsdottir et al. 2002; Mannion and Rothburn 1990; Wood et al. 1985; Pellizzer et al. 1996), septicemia (Durand et al. 1995), necrotizing pneumonitis (Torre et al. 1990), septic arthritis (Campbell et al. 1993), cerebral abscess (Akhaddar et al. 2002), and liver abscess (Nakarai et al. 2000) have been reported. The predisposing factors include underlying disease, immunocompromised status, or early age, although liver abscess caused by *L. lactis* ssp. *cremoris* also in an immunocompetent adult has recently been reported (Antolín et al. 2004). Taking into account the extent of human exposure to dairy lactococci, these infections represent extremely rare individual cases and should not be regarded as an indication of human pathogenicity. Moreover, in older cases, the possibility of misidentification of *L. garvieae* as *L. lactis* cannot be ruled out. Consequently, the European Food Safety Authority (EFSA) has included *L. lactis* ssp. *lactis* and *L. lactis* ssp. *cremoris* in the QPS (qualified presumption of safety) list of microorganisms. This means a greatly simplified safety assessment, when these bacteria are notified to EFSA for various applications (EFSA 2009).

4.5.2 Other Lactococcal Species

The fish pathogenicity of *L. garvieae* and *L. piscium* has been already mentioned in Section 2. Especially the pathogenicity and diagnostics of *L. garvieae* has been a focus of attention since the species can also be found in clinical specimen of warm-blooded animals and is frequently found as an accidental commensal microorganism in food products. It can, in rare cases, be also associated with severe human infections even in immunocompetent hosts (Li et al. 2008 and references therein). No actual virulence factors have been identified, except for the presence of capsular structures in fish pathogenic species (Barnes et al. 2002). Menéndez et al. (2007) have identified 26 genes that are associated with the ability to grow in fish, many of them of unknown function or associated with general metabolic pathways. Whether these genes have relevance to pathogenesis in warm-blooded animals is not known.

4.6 Conclusions

Lactococci represent a relatively compact LAB genus of seven species. The dairy species *L. lactis* ssp. *lactis* and ssp. *cremoris* have a long history of safe use in established applications, and both their genetics and physiology have been extensively studied. Their future applications may expand outside the traditional food use to biotechnological production of pharmaceuticals. While the other species are currently not used as starters, some of them might have potential uses, provided that their safety can be established and that they prove to be technologically compatible with industrial processes. *L. garvieae* and *L. piscium* are known fish pathogens, the former also occasionally infecting warm-blooded animals. The mechanisms behind their pathogenic potential should be elucidated before their biotechnological applications can be seriously considered.
References


Genus Lactococcus


Genus Lactococcus


Chapter 5
Genus Lactobacillus

Rodolphe Barrangou, Sampo J. Lahtinen, Fandi Ibrahim, and Arthur C. Ouwehand

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5.1 The Taxonomic Unit Defined

Lactobacilli are microaerophilic gram-positive bacteria commonly found in a diversity of environments, including nutrient-rich dairy environments, microbial-heavy host habitats such as human mucosal surfaces, as well as natural ecological niches such as plants and soil. The genus Lactobacillus belongs to the phylum Firmicutes, the class Bacilli, and the order Lactobacillales. The multiplicity of the environmental niches of lactobacilli is reflected in the diversity and the heterogenic phylogeny of the genus. The genus comprises over a hundred different species, among
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Others many well-characterized and biologically, technologically, and commercially relevant species such as *L. acidophilus*, *L. casei*, *L. delbrueckii* subsp. *bulgaricus*, *L. plantarum*, *L. rhamnosus*, and *L. salivarius* (Kleerebezem and Vaughan 2009). The genus *Lactobacillus* is characterized by strong genetic dichotomy between the species (Canchaya et al. 2006). The phylogenetic tree of genus *Lactobacillus* is complex and is divided by other genera such as *Leuconostoc*, *Oenococcus*, and *Pediococcus*, all members of the order *Lactobacillales*. Advances in full genome sequencing have resulted in changes in the taxonomy of the genus *Lactobacillus*; in recent years many species formerly classified as lactobacilli have been reclassified and now belong to other genera, including *Atopobium*, *Carnobacterium*, *Leuconostoc*, *Oenococcus*, and *Weissella*.

### 5.2 Typical Ecological Niches

Members of the genus *Lactobacillus* occupy numerous different ecological niches in nature. Within the members of the animal kingdom from honeybees to humans, lactobacilli form a part of the natural microbiota of the host animals and occupy various niches within the host such as the gastrointestinal tract, urogenital tract, oral cavity, and skin. Lactobacilli are present in dairy environment and are particularly abundant in fermented dairy products. In addition, lactobacilli are naturally present in plants and soil. *L. plantarum* has been reported to be a dominant naturally occurring bacterial species in vegetables such as cabbage and lettuce (Yang et al. 2010a). Lactobacilli are also found in fermented nondairy foods. *Lactobacillus* species have furthermore been isolated from soil samples. Examples include *L. plantarum*, *L. paracasei* subsp. *paracasei*, and *L. brevis* (Chen et al. 2005).

In humans, lactobacilli are found throughout the gastrointestinal tract, from the oral cavity to fecal material. In the oral cavity, lactobacilli are present in saliva and in dental plaque; species include *L. rhamnosus*, *L. gasseri*, and *L. casei*, among others (Yang et al. 2010b). The lactobacilli in the oral cavity may offer protection against harmful microbes, but as acid producers they themselves may also contribute to dental erosion (Haukioja et al. 2008). Lactobacilli are relatively abundant in the gastrointestinal tract. Numerous different *Lactobacillus* species have been isolated from the gastrointestinal tract and from fecal samples. The typical species composition of the intestinal *Lactobacillus* population varies among subjects and geographical regions; examples of typical species include *L. rhamnosus*, *L. acidophilus*, *L. plantarum*, *L. paracasei*, and *L. reuteri*. While lactobacilli are frequently found in fecal samples, they are present in relatively low levels and normally represent a minor part (0.2–1.0%) of the fecal microbiota (Mueller et al. 2006). Lactobacilli are genetically well adapted for the gastrointestinal environment, as reviewed later in this chapter. Intestinal lactobacilli have several interactions with the host and have been linked with numerous health benefits; these will be reviewed in other chapters of this book.

Lactobacilli are important members of a healthy vaginal microbiome. Ravel and co-workers (2011) characterized the vaginal microbiome of 396 North American women and reported that the communities were clustered into five different groups, four of which were dominated by species of lactobacilli, namely *L. iners*, *L. crispatus*, *L. gasseri*, or *L. jensenii*, and a fifth cluster with lower proportion of lactobacilli. Lactobacilli are also naturally and frequently present in human breast milk (Collado et al. 2009). *Lactobacillus* species detected from breast milk include *L. fermentum*, *L. rhamnosus*, *L. gasseri*, and *L. salivarius* (Martin et al. 2007). In addition to natural niches of lactobacilli, members of the genus are widely used in food and feed manufacturing, and are also used commercially as health-promoting microbes (i.e., probiotics).
5.3 Lactobacilli in Food and Feed

Lactobacilli have been part of normal human diet since the days of the cavemen, when the lack of means for preservation meant that much of the stored food became naturally fermented (Bengmark 1998). Today, lactobacilli are present in many different foods and have an excellent record for safety. They are used as starter cultures in food fermentation and as probiotics—health-promoting microbes. Lactobacilli are found in many if not most fermented foods, particularly in dairy products such as yogurts and its regional varieties, cheeses, and fermented milks. Many traditional regional fermented products such as the Korean kimchi and the Caucasian kefir are fermented with lactic acid bacteria (LAB), among them strains of Lactobacillus. Lactobacilli are important starter cultures in vegetable fermentations such as sauerkraut and pickled vegetables, and are used in the making of sourdough bread. Lactobacilli are also found in meats; for example, L. sakei is used in fermented meat products and can be found naturally in fresh meat and fish (Chaillou et al. 2005). In alcoholic drinks such as beer and wine, lactobacilli may contribute to the flavor of the product but may also act as contaminants. In addition to fermented foods in human diet, Lactobacillus fermentation is also utilized in animal feeds. Species such as L. plantarum and L. buchneri are used in the production of silage, a fermented animal feed (Hu et al. 2009).

Lactobacilli are also used commercially as probiotics. Probiotics are used as dietary supplements, commonly administered as capsules or sachets, and in probiotic foods. Probiotic foods may be fermented foods such as yogurts, but they are also used in nonfermented foods and beverages such as probiotic ice cream, probiotic snacks, and probiotic juices. Clinically documented probiotic Lactobacillus strains include, among others, L. acidophilus NCFM, L. acidophilus La-5, L. casei Shirota, L. casei DN-114 001, L. rhamnosus GG, L. rhamnosus HN001, L. rhamnosus GR-1, L. plantarum 299v, and L. reuteri ATCC 55730. Probiotic lactobacilli are linked with numerous beneficial health effects, as reviewed in other chapters of this book. In addition to human probiotics, lactobacilli are also used as probiotic ingredients in feed for farm animals and companion animals. While the field of human probiotics is largely dominated by lactobacilli and bifidobacteria, animal probiotics display greater variability and many commercial animal probiotics come outside these two genera. Nevertheless, lactobacilli have been investigated and utilized as probiotics for animals such as pigs, poultry, and cattle (Gaggia et al. 2010).

5.4 Metabolic Characteristics

The main metabolic characteristics of LAB are provided in the Chapter 1. In this section, the main attributes of the metabolic pathways of the genus Lactobacillus are summarized only briefly.

5.4.1 Carbohydrate Metabolism

5.4.1.1 Sugar Transport

Free sugars can be transported through either permease systems or an inducible specific phosphotransferase system (PTS). The transport of glucose, fructose, mannose, mannitol, galactose, and lactose can occur through the PTS system to end up as glucose 6-P (Kandler 1983; Monedero et al. 2007; Yebra et al. 2006). In a strain of L. casei, the PTS seems to be the main mechanism for transporting fructose, mannose, mannitol, sorbose, sorbitol, cellobiose, and lactose (Viana et al. 2000). Free pentoses are taken up by specific permeases and then converted to xylulose 5-phosphate.
to enter the pentose phosphate pathway and end with heterolactic fermentation. Twenty-five complete PTS sugar transport systems were identified in *L. plantarum* WCFS1, reflecting its efficient adaptive capacity (Kleerebezem et al. 2003).

### 5.4.1.2 Sugar Fermentation

As all LAB, species that make up the genus *Lactobacillus* are chemoheterotrophic, which means they obtain their energy for maintenance and synthesis of macromolecules solely from substrate-level phosphorylation (i.e., fermentation). Glycolysis is the most common pathway, but the pentose–phosphate pathway is also utilized in many species (see Section 1.3.1 in Chapter 1). The first step of fermentation pathways is the phosphorylation of free sugars. The phosphorylation of glucose in most species occurs through the ATP-dependent glucokinase reaction, while in some species it occurs via the PTS (Kandler 1983). Homolactic fermentation commonly occurs via glycolysis while heterolactic fermentation occurs via the pentose phosphate pathway, but homofermentative lactobacilli can also use the pentose phosphate pathway when metabolizing certain substrates. *Lactobacillus* species occur within all three groups of LAB when classified on the basis of the mode of sugar fermentation: species belonging to group I, the obligatory homofermentative species, ferment sugars through glycolysis to yield lactic acid as the end product. Species that belong to this group can switch from homofermentative fermentation to heterofermentative fermentation under some circumstances. Group II lactobacilli include the facultative heterofermentative species, which ferment sugars to yield lactic acid as the major end product plus ethanol and CO$_2$ in equimolar amounts if no other electron acceptor is available. Group III species are obligatory heterofermentative species, which can use both glycolysis and the pentose phosphate pathway. Examples of the species belonging to different groups are shown in Table 5.1.

### 5.4.2 Alternative Pyruvate Metabolism, Diacetyl Formation

Under anaerobic conditions, pyruvate generated either through glycolysis or the pentose phosphate pathway acts as an elector acceptor to regenerate NAD$^+$. This process leads to the formation of lactic acid regardless of the type of the fermentation pathway. However, there exist several alternative fates for pyruvate under various circumstances (see Chapter 1). For a different route to take place, there must be either excess pyruvate in the medium or an additional electron acceptor either formed by a different pathway or intentionally added to the growth medium. For instance, adding an exogenous electron acceptor changed the end-products profile for *L. plantarum* generating

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**Table 5.1 Mode of Sugar Fermentation in *Lactobacillus* Species**

<table>
<thead>
<tr>
<th>Homofermentative</th>
<th>Facultative Heterofermentative</th>
<th>Obligatory Heterofermentative</th>
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<tr>
<td><em>L. acidophilus</em></td>
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<td><em>L. delbrueckii</em></td>
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<td><em>L. salivarius</em></td>
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<td><em>L. plantarum</em></td>
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<td><em>L. pontis</em></td>
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acetate at the expense of lactate (Ganzle et al. 2007; Yamazaki et al. 2002). Examples of pyruvate
alternative electron acceptors include O₂, citrate, and fructose (Zaumuller et al. 2006).

Excess pyruvate can occur naturally in the medium if citrate exists, as in milk and fruits.
Fermentation of citrate occurs concomitantly with glycolysis or the pentose phosphate pathway,
producing pyruvate, and then spares the pyruvate required as electron acceptor to regenerate
NAD⁺ and leads to changes in its fate to produce formate, acetate, ethanol, and the C4 aroma
compounds (diacetyl, acetoin, 2,3-butanediol) in addition to lactic acid (Hugenholtz 1993). These
aromatic compounds and in particular the diacetyl aroma is of significant interest in the dairy
industry. Diacetyl is responsible for the characteristic aroma and flavor of butter. It can also be
found, undesirably, in other products such as wine and beer (Bartowsky and Henschke 2004).

5.4.3 Proteolytic System and Peptide Utilization

The proteolytic system is essential for bacterial growth. All species belonging to the genus
Lactobacillus require at least some amino acids depending on the specific species or strain. Some
species such as L. plantarum require only 3 amino acids while others such as L. acidophilus require
14 amino acids. Consequently, they have a functioning proteolytic system to acquire their amino
acids from the growth medium or their natural habitats. Proteolytic systems consist of protein-
ases, transport systems, and peptidases. The proteinases are secreted extracellularly to hydrolyze
proteins into oligopeptides, which are then taken up into the cell by transporters, to be further
degraded by intercellular peptidases (Christensen et al. 1999; Fadda et al. 1998). Lactobacillus spe-
cies generally exhibit low proteolytic activity. Nevertheless the characteristics of proteolytic activ-
ity have been described for several strains belonging to various species, including L. bulgaricus, L.
helveticus, L. casei, L. paracasei, L. fermentum, and L. acidophilus (El-Ghaish et al. 2010; Oneca
et al. 2007; Oberman and Jamroz 1978; Reinheimer et al. 1990), and the proteolytic system has
recently been characterized genetically and biochemically for some species (L. plantarum and L.
sanfranciscensis) (Vermeulen et al. 2005). The level and specificity of proteolytic activity differs
between species of Lactobacillus and varies even among the strains of the same species (Oberg
et al. 2002; Pereira et al. 2001); some strains can have even 15 times higher activity than others
(Awad et al. 2001). The major factors affecting the proteolytic activity of Lactobacillus strains are
pH, temperature, metal ions, and the presence of inhibitors (Abraham et al. 1993; Moon and

The effect of the proteolytic activity of 21 strains of L. helveticus and L. bulgaricus on the func-
tional properties of Mozzarella cheese showed different patterns of casein digestion (Oberg et al.
2002). There have not been extensive studies to compare the proteolytic activity among different
genera of LAB, but Tobiassen and co-workers (1997) found that strains belonging to Lactobacillus
species exhibited lower activity than those belonging to Lactococcus, but higher activity than pro-
pionibacteria (Tobiassen et al. 1997). Proteolytic systems contribute to the biochemical changes
occurring during the ripening of various fermented dairy and nondairy food products, leading to
the production of bioactive peptides with immunological and health effects (Matar et al. 2001).
They are also responsible for the organoleptic properties of the end products. The functional prop-
erties (body, texture, melt, and stretch) of Mozzarella cheese depend on the proteolytic system,
which has been shown to be strain dependent (Oommen et al. 2002). The proteolytic activity
of L. plantarum strains may have a role in preventing Clostridium botulinum toxin formation in
refrigerated foods (Skinner et al. 1999). Recent work suggests that the proteolytic activity of some
strains of L. bulgaricus can help in efforts to develop hypoallergenic dairy products as they have
the capacity to degrade the whey protein β-lactoglobulin (Pescuma et al. 2011).
5.5 Genomics of Lactobacilli

The genome sequence and content of lactobacilli reflect the diversity of environmental niches in which they occur. As the amount of information regarding the genomes of lactobacilli has increased, several studies have provided novel insights into the phylogenetic relationships of these organisms, and the functional diversity of various species. In the genomics era, access to genome sequencing platforms and in silico analyses of genomic data has provided powerful tools that provide critical insights into phylogenetic relationships of organisms, as well as the ability to correlate variable genetic content with phenotypic idiosyncrasies. Moreover, as these tools further develop, metagenomic sampling of complex ecological environments allows us to better characterize the microbial content and diversity of habitats such as the human microbiome (urogenital tract, oral cavity, skin and mucosa, gastrointestinal tract). These complex datasets can then be combined with reference genomes to unravel bacterial content and diversity, notably in important ecological surveys and clinical studies. Genetic features of LAB in general are reviewed in Chapter 2.

5.5.1 Genomic Features and Phylogeny

Genetically, lactobacilli belong to phylum Firmicutes, class Bacilli, order Lactobacillales (Makarova et al. 2006). A large diversity of Lactobacillus genomes have been published to date, covering the three main phylogenetic clusters of the genus, namely the acidophilus complex, the salivarius cluster, and the casei group (Table 2.1 in Chapter 2). Interestingly, the Lactobacillales tree is paraphyletic and the Pediococcus, Oenococcus, and Leuconostoc genera are in-between Lactobacillus branches (Makarova et al. 2006; Canchaya et al. 2006). This renders the phylogeny of the Lactobacillus genus complex, and subject to discussions and differences of opinion among the scientific community. Several genome studies have provided insights into phylogenetic relationships, species genetic idiosyncrasies, and important functional properties. Up to date, 24 Lactobacillus genomes across 16 species have been fully sequenced (see Chapter 2, Table 2.1), and an additional 56 drafts are in progress. Historically, lactobacilli were split across three phylogenetic groups, namely L. casei–Pediococcus, Leuconostoc, and L. acidophilus (Claesson et al. 2007; Nicolas et al. 2007; Schleifer and Ludwig 1995). However, further and more recent studies have further established these three groups and the species they encompass, and currently, the groups may be defined as the L. casei group, the salivarius cluster, and the acidophilus complex, respectively. The acidophilus complex includes a group of lactobacilli related to L. acidophilus, including L. johnsonii, L. gasseri, L. iners and L. bulgaricus. Overall, there is relatively high homology between species within the acidophilus complex, as determined by multilocus sequencing typing (MLST), microarray content comparisons, and genome comparisons (Berger et al. 2007). Within the acidophilus complex, L. delbrueckii stands out with a particularly high GC content (49.7%), notably peculiar at the third codon position, with a GC3 content of 65%, nearly 40% higher than those of L. acidophilus and L. johnsonii (Nicolas et al. 2007). Although synteny can be observed among members of the same phylogenetic group, notably within the acidophilus complex (van de Guchte et al. 2006), there is no synteny across phylogenetic groups (Canchaya et al. 2006). The lack of overall genome content and architecture is consistent with both the large variability observed in genome size, the various environments in which lactobacilli are found, and their different functionalities. Indeed, lactobacilli are widely used in the dairy industry, given their ability to acidify milk and develop desirable texture- and flavor-based organoleptic attributes in yogurt and cheese. Other strains are used as probiotics in various commercial applications and clinical settings due to their health-promoting functionalities, notably with regard to immunomodulation properties and intestinal balance.
The casei group is most closely related to the acidophilus complex, as opposed to the helveticus cluster (Zhang et al. 2011). The dichotomy between the acidophilus complex and the casei group on one hand, and the salivarius cluster on the other is further supported by the closer relatedness of the latter to *Oenococcus* and *Pediococcus*, and to a lesser extent, *Leuconostoc* (Zhang et al. 2011; Makarova et al. 2006).

Typically, the genomes of lactobacilli are low in GC%, which renders the identification of genes acquired by horizontal gene transfer (HGT) relatively easy when regional fluctuations in GC content are observed in genomes. The genomes are relatively small (1.3–3.3 Mbp), with 82–90% coding density, and generally encode a broad repertoire of nutrient transport and catabolism for fermentation of carbohydrates primarily into lactic acid (Makarova et al. 2006; Canchaya et al. 2006).

5.5.2 Genomic Content and Important Functionalities

Interestingly, the genome size diversity within the *Lactobacillus* genus reflects its functional polymorphism and the various environmental niches in which various species occur. Indeed, lactobacilli occur in a variety of environments including meats (Chaillou et al. 2005) and fish (Chaillou et al. 2005). Large genomes are likely derived from extensive gene acquisition through HGT, which provides the ability to grow on a variety of substrates in competitive environments with a scarcity of resources. Also, many new genes emerged through gene duplication and divergence in *L. plantarum* and *L. casei* (Makarova et al. 2006).

Lactobacilli with relatively large genomes such as *L. plantarum* notably encode numerous enzymes involved in the uptake and catabolism of carbohydrates, as well as the enzymatic machinery responsible for the biosynthesis of amino acids, nucleotides, fatty acids, and cofactors that are lacking in numerous other *Lactobacillus* species with smaller genomes (Boekhorst et al. 2004). Also, numerous cell envelope and extracellular proteins have been uniquely detected in *L. plantarum* (Siezen et al. 2010).

In contrast, lactobacilli with relatively small genomes include *L. johnsonii*, which lacks genes encoding the enzymatic machinery involved in biosynthetic pathways for amino acid, purine, and cofactor genesis (Pridmore et al. 2004). This lack of biosynthetic capabilities is usually compensated by a larger repertoire of transport systems that allow uptake of nutrients such as amino acids and carbohydrates (Pridmore et al. 2004). Indeed, several fastidious lactobacilli are unable to synthesize many amino acids, notably *L. salivarius*, which is prototrophic for 18 amino acids (Claesson et al. 2006). Also, the genome sequence of *L. bulgaricus* reveals extensive reduction, which is linked to the adaptation from a plant habitat to a rich-milk environment where this species benefits from proto-cooperation with *Streptococcus thermophilus* (van de Guchte et al. 2006).

Small genomes are likely derived from simplification by extensive deletions, which provide the ability to compete and grow quickly in rich environments with excess resources such as milk. This metabolic simplification for small genomes specifically adapted to milk applies to *L. helveticus* (Callanan et al. 2008). Interestingly, this also applies to the other domesticated dairy bacterium *S. thermophilus* (Makarova et al. 2006). The smallest *Lactobacillus* genome reported to date is that of *L. iners*, with 1.3 Mbp, which has undergone extensive gene loss (Macklaim et al. 2011).

In species where multiple genomes sequences are available, intraspecies genetic diversity can be assessed, and hypervariable regions can be identified to mine strain-specific functionalities. Hypervariable regions commonly found in the genomes of lactobacilli include insertion sequences (IS) (van de Guchte et al. 2006), restriction–modification (R/M) systems (van de Guchte et al. 2006), phage-related genes (Canchaya et al. 2006), clustered regularly interspaced
short palindromic repeats (CRISPR) loci (Horvath et al. 2009), transposons, plasmids, and genes encoding exopolysaccharides. Indeed, IS can account for a large proportion of genome evolution, as shown in *L. helveticus*, where 213 IS elements are found, nearly 10 times more than in other lactobacilli (Callanan et al. 2008). This relative variance is also found in phage-related genes, which can range from 18 to 167 across lactobacilli species (Canchaya et al. 2006). Within the homogeneous acidophilus complex, variable content primarily consists of mobile genetic elements and evidence of HGT was provided for prophages and *eps* genes (Berger et al. 2007).

From a genomic standpoint, plasmids commonly account for hypervariable content, and a few lactobacilli carry plasmids. Notably, the *L. salivarius* genome sequencing project revealed the presence of two small plasmids, and a 242 kb megaplasmid (Claesson et al. 2006). Also, analysis of genomic diversity via comparative genomic hybridization and MLST within the *L. helveticus* species has identified 18 major regions of genomic hypervariability, which include exopolysaccharide clusters, as well as other previously identified hypervariable content, notably CRISPR, plasmids, and transposases (Raftis et al. 2011). Within the *L. casei* species, comparative analyses between BL23 and ATCC 334 have identified variable genomic islands that include prophages, CRISPR, and bacteriocins, as well as lifestyle adaptation islands that include genes involved in carbohydrate utilization (Cai et al. 2009).

Overall, intraspecies genomic conservation is relatively high in lactobacilli, with 17–24% genetic divergence characterized in *L. salivarius* (24%; Raftis et al. 2011); *L. plantarum* (20%; Molenaar et al. 2005), *L. johnsonii* (17%; Berger et al. 2007), and *L. casei* (Cai et al. 2009). Interestingly, within the *L. sakei* species, genome size can vary by 25% (Chaillou et al. 2009). This is consistent with an extensive study of genomic variability in *L. plantarum* establishing that the core genome across 40 strains accounts for up to 74% of the genome (Siezen et al. 2010).

Comparative analyses of *L. helveticus* gene expression during growth in milk versus synthetic medium identified a series of genes involved in milk utilization, notably proteinases, peptidases, and oligopeptide transporters, in addition to lactose utilization genes (Smeianov et al. 2007). Likewise, genomic features within the *L. helveticus* genome have been identified that substantiate its use as a flavor adjunct in cheese starter cultures (Slattery et al. 2010). Notably, multiple proteinases (PrtP), amino acid and peptide transporters (DtpT), and peptidases (PepN, PepC, PepA, PepE) involved in proteolysis have been identified (Slattery et al. 2010).

Additionally, genetic features that encode the enzymatic machinery involved in important fermentative and ripening functionalities, texture attributes, and flavor-enhancing molecules are commonly found in dairy lactobacilli, notably *L. casei*.

In dairy cultures, given the occasional starter culture exposure to predatory bacteriophages, it is common to observe a broad and variable array of genes that provide phage resistance and viral defenses, notably R/M systems, abortive infection, and CRISPR, which together with CRISPR-associated *cas* genes form the CRISPR/Cas system (Horvath and Barrangou 2010). Indeed, CRISPR/Cas systems play a critical role in providing phage resistance in dairy cultures (Barrangou et al. 2007), and are widely distributed in lactobacilli (Horvath et al. 2009), where multiple systems occur in a large variety of species. Additionally, type I and type III R/M systems are commonly associated with dairy lactobacilli (O’Sullivan et al. 2009).

The ability to resist a variety of physical and chemical stresses is likely responsible for the survival of lactobacilli in hostile environments. Notably, the psychrotrophic ability to resist salt may be responsible for survival of *L. sakei* in fish through the curing process (Chaillou et al. 2005). Likewise, redox and antioxidant activities, linked to NADH oxidase and manganese-dependent superoxide dismutase in *L. sakei*, could be involved in robustness during food processing (Chaillou et al. 2005).
In contrast to fermentative lactobacilli, a wide array of *Lactobacillus* strains is commonly used as probiotics due to their suggested inherent health-promoting functionalities. The genomic content of these strains reflects the competitive environments in which they thrive, such as the human gastrointestinal tract, where their survival heavily relies on their ability to withstand stress and catabolize indigestible dietary compounds. The ability to survive intestinal passage partially relies on resistance to mammalian bile salts. Accordingly, genes responsible for bile salt degradation, which include bile salt hydrolases (*bsh*), are commonly found in intestinal lactobacilli (Kleerebezem et al. 2010), such as *L. johnsonii* (Pridmore et al. 2004), *L. plantarum* (Lambert et al. 2008), *L. gasseri* (Azcarate-Peril et al. 2008), and *L. acidophilus* (Altermann et al. 2005). Both *bshA* and *bshB* have been associated with gut organisms (O’Sullivan et al. 2009). Likewise, *bsh* genes have been correlated with bile salt resistance in *L. acidophilus* (McAuliffe et al. 2005) and *L. plantarum* (Lambert et al. 2008; Kaushik et al. 2009), notably through the ability to deconjugate glycine or taurine from bile salts such as glycolic acid. Also, intestinal passage survival relies heavily on acid stress resistance, as documented in *L. acidophilus* with two-component regulator-orchestrated stress response (Azcarate-Peril et al. 2004, 2005). Also, the ability of some lactobacilli to produce bacteriocins may be involved in competitive exclusion, as exemplified by the pediocin present in *L. casei* (Makarova et al. 2006). Likewise, the ability to form biofilm and/or cellular aggregation may be involved in the host colonization process, as proposed for *L. sakei* (Chaillou et al. 2005).

The ability to adhere to and interact with host epithelia and mucosal surfaces has been extensively associated with immune function in a variety of probiotic lactobacilli. Large and idiosyncratic cell surface proteins, including fimbriae, potentially involved in adherence to human glycoproteins, mucin, and fibronectin, have been implicated in host cell attachment. Specifically, mucus binding proteins encoded by *mub*, and fibronectin binding proteins encoded by *fbp* have been implicated in human epithelial cell adherence in *L. acidophilus* (Buck et al. 2005) and *L. plantarum* (Kaushik et al. 2009). Likewise, S-layer proteins have been implicated in *L. acidophilus* adherence of epithelial cells (Buck et al. 2005), and its ability to regulate dendritic and T-cell functions (Konstantinov et al. 2008). In *L. rhamnosus*, the SpaC pilin was implicated in binding to mucus, and may be involved in persistence in the human gastrointestinal tract in clinical studies (Kankainen et al. 2009).

### 5.5.3 Lactobacillus Genomics—Outlook

Genomic information has also set the basis for further studies that investigate the probiotic mechanism of action, and the molecular interplay between lactobacilli and human health, notably with regard to modulating the immune response in human mucosa and epithelia for clinical applications. Notably, several studies are focusing on the identification and characterization of candidate probiotic effector molecules involved in conferring health benefits to the host, primarily via molecular interactions between cell surface proteins (Kleerebezem et al. 2010). Indeed, lactobacilli have been repeatedly associated with or implicated in human health benefits such as gut flora shifts (Cox et al. 2010) and vaginal health (Hummelen et al. 2010), and serve as vaccine adjuvants through the activation of dendritic cells and polarization of T cells (Mohamadzadeh et al. 2005; Meijerink et al. 2010). Also, functional genomic studies have correlated particular genes with relevant phenotypes in *L. acidophilus*, as it relates to adherence to epithelial cells (Buck et al. 2005), oxalate catabolism (Azcarate-Peril et al. 2006), acid resistance (Azcarate-Peril et al. 2004, 2005), uptake of compatible solutes such as trehalose (Duong et al. 2006), carbohydrate utilization (Barrangou et al. 2003, 2006), and bile-salt hydrolysis (McAuliffe et al. 2005).
Additionally, advances in lactobacilli genomics and the development of genetic engineering systems (Russell and Klaenhammer 2001) have provided avenues for the development of strains with improved functionalities. Notably, it was shown that *L. jensenii* engineered to express CD4 could inhibit HIV infectivity (Chang et al. 2003). Likewise, *L. acidophilus* engineered with protective cell surface antigens can serve as anthrax vaccines (Mohamadzadeh et al. 2009). Also, a recent study has established that engineered lipoteichoic acid–deficient *L. acidophilus* can influence colonic inflammation (Mohamadzadeh et al. 2011). This is consistent with a previous report showing that teichoic acid modification influences the inflammation properties of *L. plantarum* (Granette et al. 2005).

Overall, the driving forces of genome evolution in lactobacilli have been simplification by loss of ancestral genes for adaptation to rich environments, coupled with gene acquisition through HGT and duplication for adaptation to select niches. The complementation of sequencing-based technologies with microarray-based platforms, and molecular biology tools provide a toolbox that allows scientists to thoroughly delve into mechanistic and functional studies that establish a basis for strain-specific characterization, detection, engineering, and development. This allows overcoming limiting technological factors, and refocus efforts on intellectual property development, regulatory challenges, and clinical studies as to set the stage to further leverage bio-based solutions for sustainable human nutrition and health.

### 5.6 Summary

The genus *Lactobacillus* is a heterogeneous group of microaerophilic gram-positive bacteria, characterized by strong genetic dichotomy between the species. Lactobacilli are of great importance to humans; for example, they are essential members of a healthy human microbiota at various niches and serve as technologically, biologically, and functionally important components of a healthy human diet. An important ecological niche of lactobacilli is the gastrointestinal tract of animals. Lactobacilli can also be isolated from various other sources such as vaginal microbiota and breast milk, as well as from plants and soil. They are used in fermented foods and many strains are also consumed as probiotics. Based on their main sugar fermentation pathways—glycolysis and pentose phosphate pathways—the *Lactobacillus* species can be grouped into homolactic, facultative heterolactic, and obligatory heterolactic species. In addition, under different conditions, lactobacilli feature various alternative pyruvate metabolism pathways involving different electron acceptors. In general, *Lactobacillus* species exhibit low proteolytic activity, but species and strain differences are remarkable and proteolytic activity of lactobacilli is important, for example, from the perspective of dairy technology. Genomics of lactobacilli reflect the diversity of environmental niches in which they occur. Recent advancements in molecular biology have greatly benefited the research of lactobacilli and offered new information on the phylogenetic relationships of the genus and the functional diversity of various species. To date, several full genomes of lactobacilli have been sequenced, providing new insights into properties of the genus and the species within. These advances have greatly facilitated the understanding of the biological functions and technological features of lactobacilli.

### References

Genus Lactobacillus


Genus Lactobacillus


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Chapter 6

The Lesser LAB Gods: *Pediococcus, Leuconostoc, Weissella, Carnobacterium, and Affiliated Genera*

Geert Huys, Jørgen Leisner, and Johanna Björkroth

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6.1 Introduction

Although *Enterococcus*, *Lactococcus*, *Lactobacillus*, *Streptococcus*, and *Bifidobacterium* are among the best studied genera, together they only partially reflect the extremely broad taxonomic and functional diversity of the lactic acid bacteria (LAB) known to date. This chapter deals with a number of LAB genera that are less well known because of one or several reasons: (i) they have only recently been described, (ii) they comprise only a few species, (iii) they have been taxonomically confused with other LAB genera, and/or (iv) they reside in very specific or underexplored habitats, which may have important implications for their isolation. A selection of seven of these “lesser gods” with relevance to the food and health area are described here in an order that aims to reflect their historical affiliation throughout the ever-changing LAB taxonomy. The description of the first genus, *Pediococcus*, is followed by that of the genus *Tetragenococcus*, which was originally proposed to accommodate atypical salt-tolerant pediococci but is now assigned to the *Enterococcaceae*. Subsequent to the genus *Leuconostoc* are the descriptions of three other members of the *Leuconostocaceae* family, that is, *Weissella*, *Oenococcus*, and *Fructobacillus*. The chapter is closed by the description of *Carnobacterium*, the type genus of the *Carnobacteriaceae*.

6.2 Genus *Pediococcus*

6.2.1 General Characteristics

Pediococci are gram-positive, nonmotile oxidase-negative, and catalase-negative organisms occurring as spherical cells uniform in size that form tetrads via alternate division in two perpendicular directions (Günther and White 1961). In contrast to other cocci in the LAB such as leuconostocs, streptococci, or lactococci, members of the genus *Pediococcus* usually do not form chains of cells. Some strains may exhibit pseudocatalase activity on media with low carbohydrate content (Weiss 1992).
Pediococci are facultatively aerobic homofermenters that produce lactic acid as the major end product of glucose fermentation by the Embden–Meyerhof pathway to d,l-lactic acid except for strains of *Pediococcus clausenii*, which convert glucose to l(+)-lactic acid. Fructose, mannose, and cellobiose are fermented by all species. Most species are also able to ferment galactose and maltose, although it has been reported that some strains of *Pediococcus damnosus*, *Pediococcus parvulus*, and *P. clausenii* lack this ability (Simpson and Taguchi 1995; Dobson et al. 2002). Sucrose is also fermented by all species except *Pediococcus inopinatus*, *P. parvulus*, *Pediococcus pentosaceus*, and *P. clausenii*. In contrast, rhamnose, melibiose, melezitose, raffinose, inulin, and α-methyl glucoside-D are not fermented by most pediococci. Pediococci can grow at pH 5 but not at pH 9. Nitrate is not reduced and indole is not formed from tryptophan. Their peptidoglycan type is Lys-d-Asp. Additional physiological and biochemical features of pediococci have been reviewed in Holzapfel et al. (2006).

Like many other LAB pediococci also produce bacteriocins (i.e., pediocins). A majority of these bacteriocins belong to bacteriocin class IIA and contain small (less than 10 kDa) cationic proteins with antilisterial activity with an YGNGV-consensus sequence (tyrosine-glycine-asparagine-glycine-valine) in the N-terminus and a more variable hydrophobic and/or amphiphilic C-terminal part (Nes et al. 1996). Producer strains of pediocins have mainly been found in the phylogenetically and biochemically related species *Pediococcus acidilactici* and *P. pentosaceus* and, more recently, also in *P. damnosus* (Papagianni and Anastasiadou 2009).

### 6.2.2 Phylogeny and Taxonomy

Based on 16S rRNA gene sequence information, the genus *Pediococcus* is currently situated in the *Lactobacillaceae* family. More specifically, *Pediococcus* has been positioned on the *Lactobacillus casei* branch of the LAB, also referred to as the *Lb. casei–Pediococcus* group within the *Lactobacillales* order (Collins et al. 1990, 1991; Collins and Wallbanks 1992). However, comparative genomics of LAB based on phylogenetic analysis of multiple protein sequences suggests a revision of the taxonomy of the *Lactobacillales* (Makarova et al. 2006). According to these new insights, the *Pediococcus* group is a sister to the *Leuconostoc* group, which supports the paraphyly of the *Lactobacillus* genus. On the basis of their atypical phylogenetic position, a number of species originally classified in the genus *Pediococcus* have been transferred to other genera of the LAB. The halophilic species *Pediococcus halophilus* and the alkaline-tolerant species *Pediococcus urinaceaequi* have been reclassified as *Tetragenococcus halophilus* (Collins et al. 1990; Anon. 1993) and *Aerococcus urinaceaequi* (Felis et al. 2005), respectively. More recently, the atypical l(+)-lactic acid-producing species *Pediococcus dextrinus* was transferred to the genus *Lactobacillus as Lactobacillus dextrinus* on the basis of multilocus sequence analysis of the 16S rRNA gene and four protein-encoding genes (Haakensen et al. 2009).

For many decades, five species were considered to represent the taxonomic core of the genus *Pediococcus*; that is, *P. damnosus* (the type species of the genus), *P. acidilactici*, *P. pentosaceus*, *P. parvulus*, and *P. inopinatus*. As discussed above, three atypical species located at the phylogenetic periphery of this core have been allocated to neighboring genera. Triggered by the use of sequence-based screening approaches for new biodiversity in a variety of fermented food and feed environments, at least seven new *Pediococcus* species have been described in recent years; that is, *P. clausenii* (Dobson et al. 2002), *P. cellicola* (Zhang et al. 2005), *P. stilesii* (Franz et al. 2006), *P. ethanolidurans* (Liu et al. 2006), *P. siamensis* (Tanasupawat et al. 2007), *P. argentinicus* (De Bruyne et al. 2008), and *P. lolii* (Doi et al. 2009). Noteworthy, many of these new species were described based on taxonomic characterization of only one or two strains, which limits our current insights in the functional characteristics and ecological role of these organisms.
6.2.3 Habitats

The natural habitats of pediococci are highly similar to those of other LAB such as *Lactobacillus*, *Leuconostoc*, and *Weissella*; that is, in or on raw or processed foods and in the intestinal tract of animals and humans. Of all *Pediococcus* species described to date, it is clear that *P. acidilactici* and *P. pentosaceus* are most intensively studied for their ecological significance and biotechnological potential (Holzapfel et al. 2006). Both species have been isolated from a large variety of plant materials such as vegetables, fruits, and cereals. Typically these species occur in relatively small numbers on raw plant materials, but are able to grow out rapidly during spontaneous fermentation of silage, sauerkraut, beans, cucumbers, olives, and cereals upon which they often establish a stable microbial community with lactobacilli, leuconostocs, and other LAB. Many types of traditional African foods using fermented cereals such as sorghum as well as various Asian mixed-culture inocula for the production of alcoholic beverages are known to contain pediococci (Holzapfel et al. 2006). In addition to materials from plant origin, pediococci are also associated with protein-rich animal-derived foods such as raw and fermented sausages, fresh and marinated fish, and cheese.

Among other applications in the cheese, wine, and feed industry, pediococci are most commonly exploited as commercial starter cultures in the meat sector. More specifically, selected strains of *P. pentosaceus* and *P. acidilactici* are used for the production of dry sausages owing to their ability to control the development of undesired and pathogenic microbiota (Hugas and Monfort 1997). In contrast to the protective role played by several *Pediococcus* species in food fermentations, other pediococci display undesired properties as spoilers. Owing to their typical resistance to hops, *P. damnosus*, *P. clausenii*, and *P. inopinatus* have been frequently isolated from beer or detected in the brewery environment (Dobson et al. 2002; Iijima et al. 2007; Sakamoto and Konings 2003). Especially *P. damnosus* has been recognized as an important beer spoiler causing turbidity, acidic off-tastes, and adverse flavors due to diacetyl formation. It has been shown that the some strains of *P. damnosus*, commonly referred to as ropy strains, can induce viscosity in beer by the production of exopolysaccharide (EPS) glucans, which is linked to the presence of the putative glucan synthase gene *dps* (Walling et al. 2005).

Although they only represent a minor group at microbiome scale, pediococci are considered commensals of humans and several animal species, including birds, shrimps, and piglets (Holzapfel et al. 2006). Especially in poultry and shrimp industry, strains of *P. acidilactici* have been used as probiotics to promote growth performance and strengthen host defense mechanisms (Castex et al. 2010; Lee et al. 2007). *Pediococcus* strains have been isolated from saliva and fecal samples of healthy humans, but it remains unclear to what extent they can act as opportunistic etiological agents in certain patient groups. Representatives of *P. pentosaceus* and *P. acidilactici* have been recovered from a variety of clinical specimens, including blood, diarrheal stools, peritoneal fluids, abscesses, and urine, although without a clear association with the underlying clinical condition (Barros et al. 2001). Because pediococci are vancomycin resistant, they may be confused with enterococci in routine laboratories, which may have resulted in a significant underreporting of these organisms in clinical settings.

6.2.4 Identification, Typing, and Detection

At the genus level, phenotypic differentiation of pediococci from the (historically) affiliated genera *Tetragenococcus* and *Aerococcus* is based on their ability to grow under acidic conditions (pH 5) but not under alkaline conditions (pH 9) and on the production of DL-lactic acid (except for *P. clausenii*) instead of L(+)lactic acid. The latter characteristic can also be useful to separate
The Lesser LAB Gods

pediococci from *Lb. dextrinicus* (a former atypical member of the genus *Pediococcus*) in conjunction with the ability of this species to produce gas from gluconate (Holzapfel et al. 2006).

Although phenotypic identification of *Pediococcus* species has long relied on growth characteristics and sugar fermentation patterns (Holzapfel et al. 2006), this approach has lost its discriminative value over the years due to a considerable degree of intraspecies variation, the lack of reproducibility, and the steady increase in new species descriptions during the past decade. Although the use of other more advanced phenotypic methods such as protein profiling (Leisner et al. 1999) and fatty acid analysis by pyrolysis mass spectrometry (Beverly et al. 1997) has proven its value for the discrimination between the major *Pediococcus* species, none of these have gained much popularity mainly due to a lack of exchangeability.

As with most other bacterial taxa, phenotypic approaches have largely been abandoned in favor of a series of molecular tools for the reliable and accurate identification of pediococci at the species level and beyond. Several DNA fingerprinting methods have been evaluated for this purpose, including ribotyping and randomly amplified polymorphic DNA (RAPD) analysis. Of these, automated ribotyping using a RiboPrinter system has proven particularly useful to rapidly identify *Pediococcus* and other contaminants in the beer brewing process (Satokari et al. 2000). As with many DNA fingerprint methods, this method may also have the potential to discriminate pediococci at the strain level. However, for high-resolution typing and strain identification of *Pediococcus* isolates pulsed-field gel electrophoresis (PFGE) has been recommended (Simpson et al. 2006).

In recent years, several sequence-based approaches have been developed and evaluated for the identification and typing of pediococci. As a potential alternative to conventional 16S rRNA gene sequencing analysis, De Bruyne and co-workers (2008) proposed the use of a multilocus sequencing scheme using genes encoding the α-subunits of phenylalanyl-tRNA synthase (*pheS*), RNA polymerase (*rpoA*), and ATP synthase (*atpA*) to identify *Pediococcus* spp. Multilocus approaches have also been developed for typing of specific pediococcal subpopulations of interest to the food industry; restriction enzyme analysis of *rpoC*, *ldhD*, and *mle* genes has been used to study genomic polymorphisms in *P. acidilactici* in relation to pediocin-producing and nonproducing strains (Mora et al. 2000). In another study, the malolactic gene *mle* was combined with four housekeeping genes (*recA*, *rplB*, *pyrG*, and *leuS*) in a multilocus sequence typing (MLST) study of *P. parvulus* and *P. damnosus* isolates from wine fermentation (Calmin et al. 2008).

Next to the identification and typing of *Pediococcus* isolates, there is also considerable interest in the direct and culture-independent detection of pediococci in samples from food or intestinal origin. Despite earlier attempts to detect pediococcal beer spoilers using immunological approaches, mainly DNA-based methods have been used for detection of pediococci. As such, a multiplex PCR assay based on the use of 23S rRNA gene sequences was developed for rapid identification of most typical *Pediococcus* species (Pfannebecker and Fröhlich 2008). Furthermore, this method also allowed detecting contaminations with *P. parvulus* and *P. damnosus* after purification of DNA from spoilt wine samples. Walter and co-workers (2001) developed 16S rDNA-targeted group-specific primers for use in denaturing gradient gel electrophoresis (DGGE) to detect food-associated members of the genera *Lactobacillus*, *Pediococcus*, *Leuconostoc*, and *Weissella* in human feces. DGGE-based strategies have also been employed to detect *Lactobacillus* and *Pediococcus* contaminants in active dry wine yeasts (Giusto et al. 2007). To circumvent problems intrinsic to the use of the 16S rDNA gene in DGGE such as the multi-operon effect, the RNA-polymerase β-subunit encoding *rpoB* gene has been proposed as an alternative target to detect and discriminate pediococci and other LAB spoilers in fermented beverages (Renouf et al. 2006). Furthermore, the DGGE primers used in the latter study could be adapted for real-time PCR detection of pediococci and which also allowed discrimination between several *Pediococcus* species in artificial
mixtures by performing a melting curve analysis. To specifically detect ropy \( P. \text{damnosus} \) strains in wine, real-time PCR detection of the \( \text{dps} \) gene responsible for \( \beta\)-d-glucan synthesis has been developed (Delaherche et al. 2004).

### 6.3 Genus \textit{Tetragenococcus}

#### 6.3.1 General Characteristics

On the basis of biochemical and phylogenetic evidence indicating that the species \( P. \text{halophilus} \) was an atypical member of the genus \textit{Pediococcus}, Collins and co-workers (1990) proposed the genus \textit{Tetragenococcus} (Anon. 1993) to accommodate halophilic pediococci. Morphologically, tetragenococci cannot be readily distinguished from members of the genus \textit{Pediococcus}. Both genera also share a facultative aerobic homofermentative metabolism, the ability to ferment a relatively wide range of sugars and the Lys-d-Asp peptidoglycan type.

#### 6.3.2 Phylogeny and Taxonomy

Phylogenetically, the genus \textit{Tetragenococcus} is a recognized member of the family \textit{Enterococcaceae} within the order \textit{Lactobacillales} (Ludwig et al. 2009; Anon. 2010). The first described member and type species, \( T. \text{halophilus} \), harbors halophilic strains formerly known as \( P. \text{halophilus} \). Since this description, three other species have been proposed in this genus, that is, \textit{Tetragenococcus muriaticus} (Satomi et al. 1997), \textit{Tetragenococcus koreensis} (Lee et al. 2005), and \textit{Tetragenococcus solitarius} (Ennahar and Cai 2005). The latter species was first described as \textit{Enterococcus solitarius} on the basis of a single non-lactose-fermenting strain (Collins et al. 1989), but was later phylogenetically relocated and assigned to the genus \textit{Tetragenococcus}.

#### 6.3.3 Habitats

From the ecological point of view, \( T. \text{halophilus} \) and \( T. \text{muriaticus} \) have primarily been associated with habitats rich in salt and protein. Both salt-tolerant species are known to play an important role in halophilic fermentation processes such as the production of soy sauce, soy paste, brined anchovies, fish sauce, Japanese fermented puffer fish ovaries, Indonesian “terasi” shrimp paste, and fermented mustard (Justé et al. 2008b). However, \( T. \text{halophilus} \) also constitutes the dominant microbiota in concentrated sugar thick juice, a sugar-rich intermediate in the production of beet sugar. In fact, strains of this species have been associated with thick juice degradation, a process characterized by a pH shift from pH 9 to 5–6 and by an increase in reducing sugar content resulting in economic losses (Justé et al. 2008a). In addition to high-salt or high-sugar environments, the presence of \( T. \text{halophilus} \) has also been reported in pig feces (Simpson and Taguchi 1995). Virtually nothing is known about the natural habitats of \( T. \text{koreensis} \) and \( T. \text{solitarius} \), two species of which the original descriptions were based on one isolate from the traditional Korean food kimchi and a human isolate, respectively.

#### 6.3.4 Identification, Typing, and Detection

Tetragenococci can be readily distinguished from pediococci mainly by their high salt tolerance (depending on the species, from 6.5% to 25% NaCl [w/v]) and ability to grow at high pH values
up to 9.0 but not at pH 5.0 (Holzapfel et al. 2006; Lee et al. 2005). Unlike most *Pediococcus* species, *Tetragenococcus* spp. produce l(+)-lactic acid instead of dl-lactic acid. Phenotypic differentiation from the phylogenetically more closely related enterococci cannot be achieved on the basis of salt tolerance as many *Enterococcus* spp. can grow in the presence of 6.5% NaCl (w/v). Instead, the inability of tetragenococci to ferment lactose may be of diagnostic value for this differentiation (Ennahar and Cai 2005). Although a series of biochemical characteristics have been described to discriminate between the four species of the genus *Tetragenococcus* (Lee et al. 2005), most studies focus on the differentiation between *T. halophilus* and *T. muriaticus*. In contrast to *T. halophilus*, strains of *T. muriaticus* are not able to grow in media without NaCl and differ in their fermentation pattern of l-arabinose, sucrose, and d-mannitol (Kobayashi et al. 2000). The latter authors also demonstrated the use of restriction enzyme analysis of 16S rDNA amplicons, partial 16S rDNA sequence analysis, and DNA–DNA hybridizations to distinguish between the two species. At the intraspecific level, it has been shown that BIOLOG, repetitive PCR fingerprinting (rep-PCR) and RAPD fingerprinting can be applied to differentiate *T. halophilus* isolates from salt- and sugar-rich environments (Justé et al. 2008b).

### 6.4 Genus *Leuconostoc*

#### 6.4.1 General Characteristics

Leuconostocs are gram-positive, nonmotile, and asporogenous bacteria, of which cells are ellipsoidal to spherical, often elongated, and usually occur in pairs or chains (Garvie 1986). When grown on a solid medium, cells are elongated and can be mistaken for rods. True cellular capsules are not formed, but many leuconostocs produce extracellular dextran that forms an electron-dense coat on the cell surface. They are facultative anaerobic and catalase negative. Before the genome sequencing project of *Leuconostoc gasicomitatum* LMG 18811\(^T\), it was thought that no functional cytochromes are present. The sequences of four fully assembled *Leuconostoc* genomes have shown that all of them possess genes encoding cytochrome *bd* terminal oxidase and for synthesizing menaquinone. However, different from *L. mesenteroides* DSM 20343\(^T\), *L. gasicomitatum* LMG 18811\(^T\) has a functional electron transport requiring only externally supplied heme for respiration (Johansson et al. 2011). Leuconostocs cannot hydrolyze arginine and do not reduce nitrate. They are nonproteolytic and nonhemolytic. Indole is not formed. Although growth may occur at pH 4.5, leuconostocs prefer an initial medium pH of 6.5. The optimal growth temperature is between 20°C and 30°C. The psychrotrophic species *L. carnosum*, *L. gasicomitatum*, *L. gelidum*, and *L. inhae* grow at refrigerated temperatures, growth of some strains has been detected even at 1°C.

All leuconostocs need rich media supplemented with growth factors and amino acids. Growth on agar media such as the de Man–Rogosa–Sharpe (MRS) medium is poor without creating anaerobic atmosphere. Colonies develop usually after 3 to 5 days, are smooth, round, grayish white, and less than 1 mm in diameter. Major metabolic routes can be mined from the four *Leuconostoc* species with completely assembled genomes, *L. citreum* KM20 (Kim et al. 2008) and *L. mesenteroides* ATCC 8293\(^T\) (Makarova et al. 2006), *L. kimchii* IMSNU11154 (Oh et al. 2010), and *L. gasicomitatum* LMG 18811\(^T\) (Johansson et al. 2011; accession no. FN822744). All of them contain a wide set of genes involved in uptake of sugars, citrate, and amino acids. The genomes include the genes for the phosphoketolase pathway and three alternative pathways for pyruvate utilization by lactate dehydrogenase, pyruvate dehydrogenase, and α-acetolactate synthase. Glucose is fermented to equal amounts of d(−)-lactic acid, CO\(_2\), and ethanol or acetate.
6.4.2 Phylogeny and Taxonomy

The genus *Leuconostoc* belongs to the *Leuconostocaceae* family within the order *Lactobacillales*, together with the genera *Fructobacillus*, *Oenococcus*, and *Weissella*. Phylogenetic analyses of the 16S rRNA gene led to the subdivision of *Leuconostoc* in three distinct lineages: the genus *Leuconostoc sensu stricto*, the *Leuconostoc paramesenteroides* group, and *Leuconostoc oenos* (Martinez-Murcia and Collins 1990; Martinez-Murcia et al. 1993). Furthermore, polyphasic taxonomy studies have been leading to several taxonomic revisions within the group *Leuconostoc*. A new genus, *Weissella* (Collins et al. 1993), was described to accommodate members of the so-called *L. paramesenteroides* group (including *L. paramesenteroides* and some atypical, heterofermentative lactobacilli). In addition, *L. oenos* has been reclassified as *Oenococcus oeni* (Dicks et al. 1995). More recently, some atypical leuconostocs of plant origin, including *Leuconostoc durionis*, *Leuconostoc ficulneum*, *Leuconostoc fructosum*, and *Leuconostoc pseudoficulneum* were assigned to the new genus *Fructobacillus* (Endo and Okada 2008). After these reclassifications, the genus *Leuconostoc sensu stricto* includes 12 validly published species names (Table 6.1) with *L. mesenteroides* as the type species.

With the exception of *L. fallax*, 16S rRNA gene sequence similarities among the type strains of *Leuconostoc* spp. are high, varying from 97.3% to 99.5% (Björkroth and Holzapfel 2006). In addition to the 16S rRNA gene, *atpA*, *dnaK*, *pheS*, *recN*, and *rpoA* loci have been analyzed. The phylogenetic trees of *pheS*, *rpoA*, and *atpA* loci proved to offer discriminatory power for differentiation.

### Table 6.1 *Leuconostoc* Species with Validly Published Names

<table>
<thead>
<tr>
<th>Species</th>
<th>First Source of Identification</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. carnosum</em></td>
<td>Vacuum-packaged, cold-stored meat</td>
<td>Shaw and Harding (1989)</td>
</tr>
<tr>
<td><em>L. citreum</em></td>
<td>Honey-dew of rye ear</td>
<td>Farrow et al. (1989)</td>
</tr>
<tr>
<td><em>L. fallax</em></td>
<td>Sauerkraut</td>
<td>Martinez-Murcia and Collins (1991)</td>
</tr>
<tr>
<td><em>L. gasicomitatum</em></td>
<td>Modified atmosphere packaged marinated broiler</td>
<td>Björkroth et al. (2000)</td>
</tr>
<tr>
<td><em>L. gelidum</em></td>
<td>Vacuum-packaged, cold-stored meat</td>
<td>Shaw and Harding (1989)</td>
</tr>
<tr>
<td><em>L. holzapfelli</em></td>
<td>Ethiopian coffee fermentation</td>
<td>De Bruyne et al. (2007)</td>
</tr>
<tr>
<td><em>L. inhae</em></td>
<td>Kimchi</td>
<td>Kim et al. (2003)</td>
</tr>
<tr>
<td><em>L. kimchii</em></td>
<td>Kimchi</td>
<td>Kim et al. (2000)</td>
</tr>
<tr>
<td><em>L. lactis</em></td>
<td>Dairy product</td>
<td>Garvie (1960)</td>
</tr>
<tr>
<td><em>L. mesenteroides</em></td>
<td>Slime formed from sugar beet solution</td>
<td>Garvie (1986)</td>
</tr>
<tr>
<td><em>L. palmae</em></td>
<td>Palm wine</td>
<td>Ehrmann et al. (2009)</td>
</tr>
<tr>
<td><em>L. pseudomesenteroides</em></td>
<td>Cane juice</td>
<td>Farrow et al. (1989)</td>
</tr>
</tbody>
</table>

*L. argentinum* has been reclassified as a later synonym of *Leuconostoc lactis* (Vancanneyt et al. 2006).
of species within the genus *Leuconostoc*, and were roughly in agreement with 16S rRNA gene-based phylogeny (Ehrmann et al. 2009; De Bruyne et al. 2007). Comparative sequencing of the additional phylogenetic markers *dnaK* and *recA* confirmed the 16S rRNA gene tree topology in the study describing *L. palmae* (Ehrmann et al. 2009). Arahal et al. (2008) tested the discriminatory power of the *recN* locus and concluded that, used either alone or in combination with 16S rRNA encoding gene sequences, *recN* can serve as a phylogenetic marker as well as a tool for species identification. Congruence of evolutionary analyses inside the *Leuconostoc–Oenococcus–Weissella* clade has been assessed by comparative phylogenetic analyses of 16S rRNA, *dnaA*, *gyrB*, *rpoC*, and *dnaK* housekeeping genes (Chelo et al. 2007). Phylogenies obtained with the different genes were in overall good agreement, and a well-supported, almost fully resolved phylogenetic tree was obtained when the combined sequence data were analyzed in a Bayesian approach.

### 6.4.3 Habitats

Leuconostocs are associated with plants and decaying plant material. They have been detected in green vegetation and roots (Hemme and Foucaud-Scheunemann 2004; Mundt 1967) and in various fermented vegetable products, such as cucumber, kimchi, cabbage, and olives (Kim and Chun 2005; Mäki 2004). In addition to plant-originated material, leuconostocs are frequent in foods of animal origin, including raw milk and dairy products, meat, poultry, and fish (Kim and Chun 2005; Björkroth and Holzapfel 2006). However, healthy warm-blooded animals, including humans, are rarely reported to carry *Leuconostoc* in the microbiota of their gut or mucous membranes. Noteworthy, leuconostocs have been recovered from the intestines of fish (Williams and Collins 1990).

The species *L. carnosum*, *L. gasicomitatum*, and *L. gelidum* have often been associated with food spoilage (Ringø et al. 1998). Some modified atmosphere packaged meat and vegetable-based foods have been prone to *Leuconostoc* spoilage; manifested as bulging of the packages, off-odors and smells, and color changes.

Although leuconostocs are not a risk for healthy individuals and are considered “generally regarded as safe” organisms (Schillinger et al. 2006), some *Leuconostoc* species have been associated with human infections. However, most of the patients involved in these infections had received vancomycin therapy, had an underlying disease, or were premature babies. Noteworthy, all leuconostocs are intrinsically resistant to vancomycin and other glycopeptide antibiotics (Buu-Hoi and Branger 1985; Huygens 1993; Orberg and Sandine 1984; Elisha and Courvalin 1995).

### 6.4.4 Identification, Typing, and Detection

Leuconostocs grow well on MRS and other media designed for LAB (de Man et al. 1960), but there are no selective media for their detection. Anaerobic atmosphere is recommended for obtaining good growth on solid media. An incubation temperature of 25°C is recommended if the species to be cultivated are not known. The psychrotrophic species *L. carnosum*, *L. gasicomitatum*, *L. gelidum*, and *L. inhae* do not grow at 37°C, and some strains may also not grow at 28°C.

Identification of leuconostocs to the species level is challenging with biochemical tests. Carbohydrate fermentation profiles vary considerably among *Leuconostoc* species, and the profiles are often strain dependent. Therefore, commercial carbohydrate fermentation reaction-based series do not provide reliable identification results (Kulwichit et al. 2007). At the genus level, leuconostocs can be distinguished from homofermentative streptococci and enterococci on the basis of their heterofermentative glucose metabolism using, for example, a tomato-containing medium
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(Gibson and Abdel-Malek 1945). The other heterofermentative LAB, such as some *Lactobacillus* and *Weissella* spp., are mainly rod shaped. The differentiation between leuconostocs and coccoid *Weissella* provides the main challenge since both genera are heterofermentative. This has led to the use of molecular approaches. A genus-specific PCR method (Schillinger et al. 2008) was developed for differentiation between strains of *Leuconostoc* and *Weissella*.

In addition, numerical analysis of macromolecule patterns (Elliott and Facklam 1993; Björkroth et al. 1998) and gene sequencing (De Bruyne et al. 2007; Nour 1998) have been applied to identify leuconostocs. An example of applying various methods as a polyphasic identification approach within the genus *Leuconostoc* was given by De Bruyne et al. (2007). The latter authors used physiological and biochemical tests together with 16S rRNA, *pheS*, *rpoA*, and *atpA* housekeeping gene analyses and numerical taxonomy analyses of fluorescent amplified fragment length polymorphism and whole-cell protein electrophoresis patterns.

A range of DNA-based methods has been applied to characterize leuconostocs at the strain level for typing, including ribotyping based on 16+23S rRNA gene restriction fragment length polymorphism (RFLP), PFGE (Björkroth et al. 1998; Vihavainen and Björkroth 2009), and various PCR-based methods such as RAPD (Nieto-Arribas et al. 2010). Typing methods have been applied to study *Leuconostoc* contamination at food manufacturing facilities or for the identification of specific spoilage organisms (Björkroth 1997; Björkroth and Korkeala 1997). Beneficial leuconostocs have been characterized to better understand food fermentations and to develop or improve starter cultures. Genome diversity in the genera *Fructobacillus*, *Leuconostoc*, and *Weissella* was also determined by physical and PFGE mapping (Chelo et al. 2010).

### 6.5 Genus Weissella
#### 6.5.1 General Characteristics

*Weissella* are gram-positive, nonmotile, and asporogenous short rods with rounded tapered ends, or ovoid (Collins et al. 1993; Björkroth et al. 2009). They occur in pairs or in short chains, and there is tendency toward pleomorphism in some of the species. They are catalase negative, facultatively anaerobic chemo-organotrophs, and were originally considered not to contain cytochromes. However, since many LAB have later been found to possess cytochromes and functional heme-dependent respiration, this may not be true either in case of *Weissella*.

*Weissella* ferment glucose heterofermentatively. Carbohydrates are fermented via the hexosemonophosphate and phosphoketolase pathways. End products of glucose fermentation are CO₂, ethanol, and/or acetate. Depending on the species, the configuration of the lactic acid produced is either DL- or D (–). *Weissella* have complex nutritional requirements as amino acids, peptides, fermentable carbohydrates, fatty acids, nucleic acids, and vitamins are generally required for growth. Biotin, nicotine, thiamine, and panthotenic acid or its derivatives are required. Arginine is not hydrolyzed by all species. Growth occurs at 15°C; some species grow at 42–45°C. *Weissella* have specific peptidoglycan structure based on lysine as a diamino acid, and with the exception of *Weissella kandleri* (Holzapfel and Van Wyk 1982) all contain alanine, or alanine and serine, in the interpeptide bridge. The interpeptide bridge of *W. kandleri* (Lys-L-Ala-Gly-L-Ala₂) contains glycine (Holzapfel and Van Wyk 1982). *Weissella* species are usually nonmotile (Collins et al. 1993). However, a novel motile species with peritrichous flagella, *Weissella beninensis*, was recently described (Padonou et al. 2010). *Weissella* species are known to produce various EPS. Production of dextran, the most well-known EPS formed by heterofermentative LAB, has been recorded

### 6.5.2 Phylogeny and Taxonomy

The genus *Weissella* was proposed by Collins et al. (1993), and its first members comprised species previously allocated in the genera *Leuconostoc* or *Lactobacillus*. The species *Leuconostoc paramesenteroides* (Garvie 1967), *Lactobacillus viridescens* (Niven and Evans 1957; Kandler and Abo-Elnaga 1966), *Lactobacillus confusus* (Holzapfel and Van Wyk 1982; Holzapfel et al. 1969), *Lactobacillus kandleri* (Holzapfel 1982), *Lactobacillus minor* (Kandler et al. 1983), and *Lactobacillus halotolerans* (Kandler et al. 1983) kept their specific epithets and were reclassified as *Weissella paramesenteroides*, *Weissella viridescens* (i.e., the type species), *Weissella confusa*, *W. kandleri*, *Weissella minor*, and *Weissella halotolerans*, respectively. These species were followed by inclusion of *Weissella hellenica* (Collins et al. 1993), *Weissella thailandensis* (Tanasupawat et al. 2000), *Weissella cibaria* (Björkroth et al. 2002), *Weissella soli* (Magnusson et al. 2002), and *Weissella koreensis* (Lee et al. 2002). In addition, *Weissella kimchii* was proposed by Choi et al. (2002) but was later recognized as a heterotypic synonym of *Weissella cibaria* (Ennahar and Cai 2004). In recent years, *W. ghanensis* (De Bruyne et al. 2008), *W. beninensis* (Padonou et al. 2010), and *W. fabaria* (De Bruyne et al. 2010) were described as new species of the genus *Weissella*.

The species *W. confusa*, *W. cibaria*, *W. halotolerans*, *W. hellenica*, *W. kandleri*, *W. koreensis*, *W. minor*, *W. paramesenteroides*, *W. soli*, *W. thailandensis*, and *W. viridescens* share 93.9–99.2% 16S rRNA gene sequence similarity (Björkroth et al. 2009). Four main phylogenetic branches exist based on 16S rRNA gene analyses. *W. hellenica*, *W. paramesenteroides*, and *W. thailandensis* are positioned on the same branch, as do *W. confusa* and *W. cibaria*. The two other branches are formed by *W. minor*, *W. viridescens*, and *W. halotolerans* and by *W. kandleri*, *W. soli*, and *W. koreensis*. The species *W. fabaria* (De Bruyne et al. 2010) and *W. ghanensis* (De Bruyne et al. 2008) are positioned distinct from the other species within the genus, and their type strains share 99.5% 16S rRNA gene sequence similarity. The remaining species, *W. beninensis*, has 97.3% 16S rRNA similarity with *W. ghanensis*, and shares 92–94% sequence similarity with *W. confusa*, *W. cibaria*, and *W. koreensis* (Padonou et al. 2010).

In addition to the 16S rRNA gene phylogeny, analysis with *pheS* (de Bruyne et al. 2010) and *recN* (Arahal et al. 2008) loci had been performed. Congruence of evolutionary relationships inside the *Leuconostoc–Oenococcus–Weissella* clade has been assessed by phylogenetic analyses of 16S rRNA, *dnaA*, *gyrB*, *rpoC*, and *dnaK* (Chelo et al. 2007) housekeeping genes. Phylogenies obtained with the different genes were in overall good agreement, and a well-supported, almost fully resolved phylogenetic tree was obtained when the combined data were analyzed in a Bayesian approach.

### 6.5.3 Habitats

The habitats of *Weissella* species are variable and most commonly involve fermented foods, but the sources of isolation suggest an environmental (e.g., soil, vegetation) origin. The species *W. viridescens*, *W. halotolerans*, and *W. hellenica* have been associated with meat and meat products. More specifically, *W. viridescens* may cause spoilage of cured meat due to green discoloration (Niven and Evans 1957) and it also is a prevailing spoiler of the Spanish blood sausage Morcilla de Burgos (Koort et al. 2006; Diez et al. 2009; Santos et al. 2005). This species is considered to be moderately heat resistant (Niven et al. 1954), which is not a common property for LAB. Members of *W. cibaria*, *W. confusa*, and *W. koreensis* have been detected in fermented foods of vegetable origin
(Björkroth et al. 2002; Lee et al. 2002), whereas W. confusa has been associated with Greek salami (Samelis et al. 1994), Mexican pozol (Ampe 1999), and Malaysian chili bo (Leisner et al. 1999). W. cibaria and W. confusa have also been associated with various types of sourdoughs (Galle et al. 2010; Katina et al. 2009; Scheirlinck et al. 2007; De Vuyst et al. 2002). W. ghanensis and W. fabaria were detected in traditional heap fermentations of Ghanaian cocoa bean (De Bruyne et al. 2010; De Bruyne et al. 2008). W. beninensis (Padonou et al. 2010) originates from submerged fermenting cassava. W. soli (Magnusson et al. 2002) is the only species known to originate from soil, although also W. paramesenteroides has been detected in soil (Chen et al. 2005). In addition, weissellas have been isolated from sediments of a coastal marsh (Zamudio-Maya et al. 2008) and lake water (Yanagida et al. 2007).

W. confusa is considered a member of the normal human intestinal microbiota (Stiles and Holzapfel 1997; Walter et al. 2001; Tannock et al. 2001). However, Weissella species such as W. cibaria and W. confusa have also been detected in clinical samples of human or animal origin (Björkroth et al. 2002). Strains of W. confusa have been associated with rare cases of bacteremia (Olano et al. 2001; Harlan et al. 2010; Salimnia et al. 2011; Lee et al. 2011) and endocarditis (Flaherty et al. 2003) in humans. Weissella-associated infections are mainly due to natural resistance of these species to vancomycin and are usually associated with an underlying disease or immunosuppression of the host. Next to human cases, W. confusa has also been documented as a cause of systemic infection in a healthy primate (Cercopithecus mona) (Vela et al. 2003) and unknown Weissella strains were isolated from a diseased rainbow trout in China (Liu et al. 2009).

6.5.4 Identification, Typing, and Detection

Identification of weissellas both at the genus and species level is challenging. Weissella is routinely cultured using the general growth media for LAB such as MRS medium (De Man et al. 1960), but there is no specific selective medium or enrichment method for the members of this genus. The intrinsic resistance to vancomycin may be useful in certain approaches, but does not distinguish between Weissella and Leuconostoc. It is most difficult to differentiate Weissella from Leuconostoc and heterofermentative Lactobacillus by phenotypic characteristics, for which reason most current identification approaches rely on chemotaxonomic and molecular methods. Numerical analyses of macromolecule patterns have proved useful in the identification of Weissella species. Weissella and Leuconostoc species have been distinguished by comparison of total soluble cell protein patterns (Dicks 1995; Tsakalidou et al. 1997). In addition, 16S+23S rRNA gene restriction patterns (ribotypes) have been used for differentiating W. confusa from W. cibaria and grouping the strains into species-specific clusters (Björkroth et al. 2002). Comparison of cellular fatty acid profiles of Weissella species (Samelis et al. 1998) correspond well with results recorded in other taxonomic studies, and were found valuable in the differentiation of W. viridescens, W. paramesenteroides, W. hellenica, and typical arginine-negative Weissella strains isolated from meat.

A genus-specific PCR method was developed for differentiation between the two heterofermentative LAB genera Leuconostoc and Weissella (Schillinger et al. 2008). For some Weissella taxa, species-specific sequences have been located in helix 1007/1022 of the variable region V6 in the 16S rRNA gene (Collins et al. 1993), and comparison of DNA patterns generated after restriction enzyme digests (MnlI, MseI, and BceAI) of a 725 bp 16S rDNA fragment has also been applied (Jang et al. 2002). The recN locus may serve as a phylogenetic marker as well as a tool for species identification either alone or in combination with 16S rRNA encoding gene data (Arahal et al. 2008).

Typing of weissellas has been based on the use of different macromolecules. Numerical analysis of either HindIII or EcoRI ribopatterns has been used to characterize W. viridescens from Morcilla
de Burgos sausages (Koort et al. 2006). Dextran-producing strains of *W. cibaria* and *W. confusa* originating from sourdough were characterized with repetitive-element-PCR fingerprinting using (GTG)$_5$-PCR (Bounaix et al. 2010). Genome diversity in the genera *Fructobacillus*, *Leuconostoc*, and *Weissella* was determined by physical and PFGE mapping (Chelo et al. 2010).

### 6.6 Genus Oenococcus

#### 6.6.1 General Characteristics

On the basis of their atypical acidophilic character and substantiated by 16S and 23S rRNA sequencing studies, Dicks and colleagues (1995) concluded that members of the species *L. oenos* represented a distinct phylogenetic line in the LAB that warranted its taxonomic transfer to a new genus, *Oenococcus*, as *Oenococcus oeni*. Still, oenococci share many physiological characteristics with *Leuconostoc* spp. such as a heterofermentative metabolism, the absence of arginine deiminase, and the production of predominantly d(−)-lactate from glucose. *Oenococcus* cells are typically (elongated or ellipsoid) coccoid and, at least in case of *O. oeni*, contain the Lys-L-Ser$_2$ or Lys-L-Ala-L-Ser peptidoglycan type (Björkroth and Holzapfel 2006).

#### 6.6.2 Phylogeny and Taxonomy

Phylogenetically, the genus *Oenococcus* is assigned to the *Leuconostocaceae* family of the *Lactobacillales* order (Schleifer 2009; Anon. 2010). On the basis of its outgroup position in 16S rRNA gene sequence-based phylogenetic trees, *O. oeni* has long been considered an example of a tachyletic (fast-evolving) organism, but this hypothesis was not supported by subsequent phylogenetic analyses based on multiple housekeeping genes (Chelo et al. 2007). Next to the type species *O. oeni*, the genus *Oenococcus* also comprises the nonacidophilic and non-malolactic-fermenting species *Oenococcus kitaharae*, which was proposed to accommodate isolates from a composting distilled shochu residue in Japan (Endo and Okada 2006).

#### 6.6.3 Habitats

Of all LAB associated with wine production, the ethanol-tolerant *O. oeni* is the most important due to its key role in the secondary fermentation of wine or the so-called malolactic fermentation. In the latter process, natural contaminants or selected starter cultures of *O. oeni* convert l-malic acid to l(+)-lactic acid, which causes a slight increase in the pH of the wine and contributes to its sensory properties (Bartowsky 2005). In contrast, some strains of *O. oeni* can also have adverse effects on wine quality, including the production of biogenic amines such as histamine from histidine (Bartowsky 2005). Intriguingly, the natural habitat of *O. oeni* remains unknown. Isolation of *O. oeni* isolates from wine grapes has thus far not been documented, indicating that this organism does not commonly occur on grapes or that current methods fail to detect its presence (Bae et al. 2006).

#### 6.6.4 Identification, Typing, and Detection

Phenotypically, *O. oeni* can be separated from leuconostocs and weissellas based on its acidophilic nature (growth at an initial pH of 4.8) and its ethanol tolerance (growth in media containing
10% ethanol) (Björkroth and Holzapfel 2006). At the species level, *O. kitaharae* is differentiated from *O. oeni* by its nonacidophilic character, the inability of malolactic fermentation, and of growth in broth containing 10% (v/v) ethanol (Endo and Okada 2006). Owing to the remarkable conservation of its ribosomal genes at infraspecies level, it has been argued that differentiation of individual *O. oeni* strains can only be achieved by using methods targeting minor genotypic differences such as RAPD or PFGE (Bilhère et al. 2009). The latter authors developed an MLST scheme based on the analysis of eight housekeeping genes that allowed delineating two subpopulations among a collection of 43 geographically diverse *O. oeni* strains. These results suggest that intergenic recombination plays a key role in the evolution of the *O. oeni* genome at the population level. Other molecular developments have largely concentrated on the detection and monitoring of indigenous strains or commercial starters of *O. oeni* in wines during vinification. Pinzani and colleagues (2004) designed a real-time PCR assay targeting the gene encoding the malolactic enzyme of *O. oeni*, whereas a recent study combined the use of Whatman FTA cards for DNA extraction and purification with restriction analysis of amplified 16S rDNA for the time-efficient detection of this organism during wine-making surveillance and wine quality control (Marques et al. 2010).

### 6.7 Genus *Fructobacillus*

#### 6.7.1 General Characteristics

The genus *Fructobacillus* was described to accommodate gram-positive, catalase-positive, nonmobile fructose-fermenting rods originating from fermented and nonfermented fruits, figs, cocoa, and flowers. On the basis of studies of 16S rRNA, 16S–23S rRNA intergenic spacer region (ISR), *rpoC*, and *recA* sequences, Endo and Okada (2008) concluded that members of the *Leuconostoc* species *L. durionis*, *L. ficulneum*, *L. fructosum* (basonym *Lactobacillus fructosus*), and *L. pseudoficulneum* represented a distinct phylogenetic line in the LAB that warranted their taxonomic transfer to a new genus. Their metabolic characteristics include production of lactic acid, CO₂, acetic acid, and trace amounts of ethanol by a heterofermentative catabolism of a limited number of carbohydrates with fructose as the optimal substrate. An electron acceptor is needed for dissimilation of glucose, whereas a fraction of fructose, especially in the presence of glucose, is converted to mannitol. Indeed, glucose catabolism is slow, frequently resulting in limited CO₂ production (Leisner et al. 2005). Their poor growth on enumeration media such as All Purpose Tween, MRS, and tomato juice agars that do not contain fructose as a principal carbohydrate source lead to an underestimation of their actual numbers when present (Nielsen et al. 2007; Endo et al. 2009). This is a problem relevant for many LAB associated with plants and plant materials (Ampe et al. 1999). The major contemporary importance of *Fructobacillus* is a highlight of this issue since no studies have yet been devoted to their potential biotechnological importance, such as production of bacteriocins and compounds important for sensory profiles. An enrichment broth containing fructose has been used for their isolation (Endo et al. 2009), which, together with culture independent methods, will be important to assess further their distribution and significance in foods.

#### 6.7.2 Phylogeny and Taxonomy

Phylogenetically the genus *Fructobacillus* (Endo and Okada 2008) is assigned to the *Leuconostoeae* family of the *Lactobacillales* order and consists of five species, that is, *F. durionis* (Leisner et al.

### 6.7.3 Habitats

The habitats of *Fructobacillus* spp. include banana (*F. pseudoficulneus*), cocoa (*F. pseudoficulneus*), fermented durian (*F. durionis*), figs (*F. ficulneus* and *F. pseudoficulneus*), flowers (*F. fructosus* and *F. tropaeoli*) (Holzapfel et al. 2009; Endo et al. 2009, 2010), and Mexican palm sap (*F. durionis* and *F. fructosus*) (Alcántara-Hernández et al. 2010). Their relatively small estimated chromosomal sizes (1.41–1.55 Mb) (Chelo et al. 2010) indicate loss of genes (Makarova et al. 2007), which support that their habitat range is restricted to a nutrient (e.g., fructose) rich environment. Some fermentation processes may result in loss of conditions required for their growth (Alcántara-Hernández et al. 2010).

### 6.7.4 Identification, Typing, and Detection

Phenotypically, *Fructobacillus* can be separated from leuconostocs by their poor growth with glucose as substrate if no electron acceptor is present, and by lack of ethanol production (Endo and Oakada 2008). In addition, they can be distinguished from most *Weissella* spp. by the production of D-lactic acid and lack of the arginine deiminase pathway (Björkroth et al. 2009). At the species level, carbohydrate fermentation patterns might be useful to separate at least *F. durionis* and *F. ficulneum* from the other species as they produce acid from a range of additional carbohydrates, including sucrose, D-turanose, gluconate, methyl-α-D-glucopyranoside, maltose, and trehalose. In most cases, however, positive reactions are weak or only obtained after extended incubation for 4–7 days (Endo et al. 2010). *F. durionis* can be differentiated from *F. ficulneum* by acid production from ribose (Leisner et al. 2005; Endo et al. 2010). Carbohydrate fermentation patterns do not distinguish between *F. tropaeoli*, *F. fructosus*, and *F. pseudoficulneus* (Endo et al. 2011). This is in agreement with Endo and Oakada (2008) who reported that there is in general no or limited differentiation between four of the *Fructobacillus* spp. based on carbohydrate utilization patterns, thereby diminishing their discriminative value. Opposed to *F. tropaeoli* and *F. fructosus*, *F. pseudoficulneus* is weakly positive in a number of enzyme activities (APIZYM), including butyrate esterase, myristate lipase, trypsin, and α-galactosidase (Endo et al. 2010). Matrix-assisted laser desorption/ionization time of flight mass spectrometry has been suggested as an alternative phenotypic method for species differentiation of *F. durionis*, *F. ficulneum*, *F. fructosus*, and *F. pseudoficulneus* (De Bruyne et al. 2011).

*Fructobacillus* spp. can be distinguished on the basis of their 16S rRNA, 16S–23S rRNA intergenic spacer region and the rpoC and recA sequences. DGGE with subsequent 16S rRNA gene sequencing has been used as a culture-independent method for detection of *F. pseudoficulneum* from cocoa fermentations (Nielsen et al. 2007).

### 6.8 Genus Carnobacterium

#### 6.8.1 General Characteristics

Carnobacteria are gram-positive, catalase-negative, and cold-tolerant organisms forming straight, slender, and nonmotile (except for some strains of *Carnobacterium mobile*) rods. They
usually occur singly or in pairs, and sometimes in short chains (Collins et al. 1987). Some species such as *Carnobacterium funditum* and *Carnobacterium pleistocenium* may form coccobacilli (Hammes and Hertel 2009). One report describes even the infrequent appearance of coccus-shaped cells of *C. mobile*-like clinical isolates when incubated at 37°C but not at 25°C (Hoenigl et al. 2010).

Carbohydrates are catabolized fermentatively by carnobacteria. However, respiration has been shown for *Carnobacterium maltaromaticum* in the presence of heme (Meisel et al. 1994; Hammes and Hertel 2009), and this species consumes substantial amounts of oxygen during exponential growth under aerobic conditions (Borch and Molin 1989). Pseudo-catalase activity has also been demonstrated for the majority of the *Carnobacterium* species (Ringø et al. 2002). The glycolytic pathway is present in *Carnobacterium divergens*, and production of L-lactic acid has been shown for the majority of carnobacterial species. Acetic acid, formic acid (anaerobically; Borch and Molin 1989), acetoin (aerobically; Borch and Molin 1989), and CO₂ may be produced as end products, presumably by decarboxylation/dissimilation of pyruvic acid. Production of lactic acid has not been reported for *C. pleistocenium* although acetate and ethanol were produced (Pikuta et al. 2005). Also, *C. alterfunditum* and *C. funditum* do not produce lactate from glycerol (Hammes and Hertel 2009). *C. divergens* and *C. maltaromaticum* utilize ribose and gluconic acid as substrates for growth, and acetic acid production can be substantial under aerobic conditions or if glucose is substituted for ribose as a carbohydrate source (Leisner et al. 2007; Hammes and Hertel 2009). This result indicates the possible presence of an inducible phosphoketolase and further suggests that carnobacteria are facultatively or atypical heterofermentative organisms.

Generally, *Carnobacterium* spp. ferment various carbohydrates but with a substantial amount of variation. Glucose, fructose, mannose, ribose, sucrose, and trehalose are utilized by the majority of species. *Carnobacterium jeotgali* is atypical by its narrow spectrum of carbohydrates for fermentation, omitting glucose, and ribose (Kim et al. 2009). Two *Carnobacterium* species, *C. divergens* and *C. maltaromaticum*, possess an unusual chitinolytic activity (Leisner et al. 2008) with two putative chitinases showing high amino acid sequence similarities to *Listeria* chitinases (Leisner et al. 2010). Other polysaccharides such as inulin (*C. divergens*, *Carnobacterium gallinarum*, and some *C. maltaromaticum* strains), starch (*C. pleistocenium*), and glycogen (*C. gallinarum* and *C. mobile*) may also be utilized (Hammes and Hertel 2009).

Production of bacteriocins has been extensively investigated, especially for *C. divergens* and *C. maltaromaticum*. Currently one circular, one class I, and 10 class II bacteriocins have been described, and several studies on their potential application have been reported, particularly on their inhibition of *Listeria monocytogenes* (Table 6.2; Leisner et al. 2007; Martin-Visscher et al. 2008).

The species *C. divergens*, *C. gallinarum*, *C. maltaromaticum*, and *C. mobile* possess the arginine deiminase pathway resulting in production of NH₄⁺ (Collins et al. 1987; Leisner et al. 1994a; Schillinger and Holzapfel 1995), whereas this is not the case for *C. inhibens*, *C. jeotgali*, or *C. viridans* (Jöborn et al. 1999; Collins et al. 2002; Holley et al. 2002). Information on this pathway is not available for *Carnobacterium alterfunditum*, *Carnobacterium funditum* (Franzmann et al. 1991), or *C. pleistocenium* (Pikuta et al. 2005). *C. divergens* and *C. maltaromaticum* are able to decarboxylate tyrosine to tyramine (Leisner et al. 1994b; Laursen et al. 2006) and to generate branched alcohols and aldehydes from valine, leucine, and isoleucine (Laursen et al. 2006; Leisner et al. 2007). Extracellular products arising from metabolism of other amino acids and proteolytic activity has not been reported. Some strains of *C. divergens* and *C. maltaromaticum* metabolize citric acid (Morea et al. 1999; Afzal et al. 2010).
Table 6.2
Diversity and Biological Distribution of *Carnobacterium* Species

<table>
<thead>
<tr>
<th>Species</th>
<th>Ecological Group</th>
<th>Phylogenetic Group</th>
<th>Isolation Frequency</th>
<th>Isolated From Food Products</th>
<th>Environment</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. alterfunditum</em></td>
<td>II</td>
<td>B</td>
<td>Very low</td>
<td>Shrimp product&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Deep sea, live fish, polar lake/sea</td>
</tr>
<tr>
<td><em>C. divergens</em>&lt;sup&gt;a&lt;/sup&gt;</td>
<td>I</td>
<td>A</td>
<td>High</td>
<td>Cheese, fish, meat, and shrimp products</td>
<td>Live fish, polar lake/sea, temperate zone soil and water</td>
</tr>
<tr>
<td><em>C. funditum</em>&lt;sup&gt;d&lt;/sup&gt;</td>
<td>II</td>
<td>B</td>
<td>Very low</td>
<td>Not reported</td>
<td>Polar lake/sea, live fish, sponge</td>
</tr>
<tr>
<td><em>C. gallinarum</em>&lt;sup&gt;d&lt;/sup&gt;</td>
<td>I</td>
<td>A</td>
<td>Low</td>
<td>Meat products</td>
<td>Not reported</td>
</tr>
<tr>
<td><em>C. inhibens</em>&lt;sup&gt;d&lt;/sup&gt;</td>
<td>I</td>
<td>B</td>
<td>Very low</td>
<td>Not reported</td>
<td>Live fish</td>
</tr>
<tr>
<td><em>C. jeotgali</em>&lt;sup&gt;d&lt;/sup&gt;</td>
<td>I</td>
<td>B</td>
<td>Very low</td>
<td>Korean fermented ingredient (seafood)</td>
<td>Not reported</td>
</tr>
<tr>
<td><em>C. maltaromaticum</em>&lt;sup&gt;b&lt;/sup&gt;</td>
<td>I</td>
<td>A</td>
<td>High</td>
<td>Cheese, fish and meat products</td>
<td>Deep sea, insects, live fish, polar lake/sea, temperate zone soil, and water</td>
</tr>
<tr>
<td><em>C. mobile</em></td>
<td>I</td>
<td>B</td>
<td>Intermediate</td>
<td>Meat and shrimp products</td>
<td>Live fish</td>
</tr>
<tr>
<td><em>C. pleistocenium</em>&lt;sup&gt;d&lt;/sup&gt;</td>
<td>II</td>
<td>B</td>
<td>Very low</td>
<td>Shrimp product&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Permafrost</td>
</tr>
<tr>
<td><em>C. viridans</em>&lt;sup&gt;d&lt;/sup&gt;</td>
<td>I</td>
<td>B</td>
<td>Very low</td>
<td>Meat product</td>
<td>Not reported</td>
</tr>
</tbody>
</table>


<sup>a</sup> Some strains produce one of the following bacteriocins: divercin V41, divergicin M35 (both class IIa), divergicin A, divergicin 750 (both class IIc).

<sup>b</sup> Some strains produce one or more of the following bacteriocins: carnobacteriocins BM1 and B2, piscicolin 126, piscicocin CS526 (all class IIa), carnobacteriocin A (IIc), carnocyclin A (cyclic).

<sup>c</sup> *C. alterfunditum*-like and *C. pleistocenium*-like isolates (Jaffrès et al. 2009).

<sup>d</sup> References for species descriptions are *C. alterfunditum* and *C. funditum* (Franzmann et al. 1991); *C. inhibens* (Jöborn et al. 1999); *C. jeotgali* (Kim et al. 2009); *C. viridans* (Holley et al. 2002); remaining species (Collins et al. 1987).
6.8.2 Phylogeny and Taxonomy

The genus *Carnobacterium* currently consists of 10 species (Table 6.2). Historically, the two initial species were described as nonaciduric, acetate-sensitive, and atypical heterofermentative *Lactobacillus divergens* and *Lactobacillus piscicola* (synonym *L. carnis*). Collins et al. (1987) proposed that these two species should be reclassified into a new genus, *Carnobacterium*, as *C. divergens* and *C. piscicola* together with two new species, *C. gallinarum* and *C. mobile*. 16S rDNA sequencing indicates that this genus belongs to the family *Carnobacteriaceae* within the order *Lactobacillales*. Later, *C. piscicola* was shown to be a synonym of *Lactobacillus maltaromaticus*, resulting in the proposal of the new species name, *C. maltaromaticum* (Mora et al. 2003). Since the original description of the genus, six additional species have been added (Table 6.2).

*Carnobacterium* spp. can be distinguished in two taxonomic groups, not to be confused with the ecological groups described further below in Section 6.8.3, based on 16S rDNA gene sequences (Hammes and Hertel 2009; Kim et al. 2009; Table 6.2). Two of the species belonging to group A (i.e., *C. divergens* and *C. maltaromaticum*) are found in a wide variety of habitats, whereas most of the species belonging to group B have, with the exception of *C. mobile*, been isolated only at few occasions (Table 6.2).

6.8.3 Habitats

The genome sizes of *Carnobacterium* spp. vary between 2.0 and 3.5 Mb for isolates of *Carnobacterium* sp. (AT7), *C. alterfunditum*, *C. divergens*, *C. maltaromaticum*, and *C. pleistocenium* (Daniel 1995; Pikuta et al. 2005; Leisner and Sørensen, unpublished results; www.moore.org). The upper range of these sizes, comparative among the LAB only to *Lb. casei*, *Lactobacillus plantarum*, and *Lactobacillus rhamnosus* signify an adaptive lifestyle and successful colonization of a range of habitats. It is therefore not surprising that members of this genus have been isolated from various sources. It is, however, noteworthy that they are generally absent in plants and plant materials.

The genus can be divided into ecological groups according to habitats (Table 6.2); however, only two species, *C. divergens* and *C. maltaromaticum*, are frequently encountered in a diverse range of environments, including foods. Group I species are associated with the man-made habitat of animal food and the natural habitats of fish intestine and gills, insects, marine sponges, soil, pond water, alpine permafrost, polar or deep sea, and cold, alkaline tufta columns, whereas group II appears to be mostly, but not exclusively, restricted to arctic ice lakes or pleistocenian ice (Table 6.2; Leisner et al. 2007; Hammes and Hertel 2009).

Food products that constitute a habitat for *Carnobacterium* spp. include vacuum or modified atmosphere packed, refrigerated raw or processed meat products and lightly preserved fish products, milk, and certain types of soft cheese (Leisner et al. 2007). Although *C. divergens* and *C. maltaromaticum* may have potential as protective cultures in foods, some strains appear to display undesirable properties as spoilers, where transamination, decarboxylation, and reduction of the amino acids isoleucine, leucine, and valine appear to be particularly important (Leisner et al. 2007). In addition, these two species also produce tyramine, a biogenic amine that constitutes a cause for concern regarding food safety for sensitive individuals, that is, individuals with reduced monoamine oxidase activity (Leisner et al. 2007).

Fish is a common animal reservoir of some carnobacteria, and some isolates of *C. maltaromaticum* have been found pathogenic in this regard. On the other hand, strains of this species and of *C. divergens* have also been considered as potential probiotics (Toranzo et al. 1993a,b; Ringø et
The Lesser LAB Gods

1.11

al. 2005; Kim and Austin 2008; Loch et al. 2008; Leisner et al. 2007). Although Carnobacterium spp. have rarely been associated with human clinical cases (Leisner et al. 2007), a number of putative virulence factors have been described for C. maltaromaticum ATCC 35586, including hemolysins, internalins, a putative capsule synthetic ability, and a gene related to the Listeria PrfA virulence regulator (Leisner et al. 2010). β-Hemolysis activity has been demonstrated for C. viridans, and also for a clinical C. mobile isolate (Holley et al. 2002; Hoenigl et al. 2010). A number of invertebrates, such as insects and marine sponges, are sources for C. maltaromaticum and C. funditum, respectively (Shannon et al. 2001; Li and Liu 2006).

6.8.4 Identification, Typing, and Detection

Because growth of carnobacteria is inhibited by acetate, their presence in foods has frequently been underestimated if acetate-containing media such as MRS or Rogosa agar have been used for their detection. Thus, culture-dependent methods for their identification prerequisite use of media that do not contain this compound; such as nitrite polymyxin (NP) agar or Carnobacterium-specific agar media (Wasney et al. 2001; Edima et al. 2007).

At the genus level, phenotypic differentiation of carnobacteria from the historically associated genus Lactobacillus but also from Paralactobacillus and Weissella is based on the observation that they grow well at pH 9 but not at low pH, for example, on acetate agar (Collins et al. 1987; Hammes and Hertel 2009). They may be differentiated from nonaciduric Leuconostoc spp. by the production of l-lactic acid from glucose. Leuconostoc and Weissella as well as Isobaculum and Desemzia (both belonging to the Carnobacteriaceae) can also be differentiated by not possessing meso-diaminopimelic acid in the peptidoglycan (Hammes and Hertel 2009; Holzapfel et al. 2009).

A number of phenotypic methods have been used for species identification and typing of Carnobacterium, including biochemical and physiological tests, composition of cellular fatty acids, whole-cell protein profiling, pyrolysis mass spectrometry, and Fourier transform infrared spectroscopy (Hammes and Hertel 2009; Afzal et al. 2010). Differentiation between the two most commonly encountered species, C. divergens and C. maltaromaticum, is only possible by using a large number of tests and data evaluations by numerical taxonomy (Laursen et al. 2005; Hammes and Hertel 2009). SDS-PAGE of whole-cell protein extracts is useful as a phenotypic identification method at species and strain level (Laursen et al. 2005).

Several genotypic methods have been evaluated for culture-dependent identification of Carnobacterium species, including multiplex PCR, rep-PCR, ribotyping, 16S–23S ISR-based RFLP, AFLP, PFGE, and RAPD (Leisner et al. 2008; Hammes and Hertel 2009; Afzal et al. 2010). RAPD has also been used for typing (Morea et al. 1999), whereas AFLP was not found suitable for this purpose in one study (Laursen et al. 2005). In addition, some of the methods are limited in their applicability because they require specific databases and/or they are difficult to standardize (e.g., 16S–23S ISR-based RFLP, Laursen et al. 2005). 16S rRNA gene sequencing has been used as a method per se or in support of results obtained with the other methods. However, this approach fails to differentiate between C. alterfunditum and C. pleistocenium (Pikuta et al. 2005; Hammes and Hertel 2009). To date, sequencing of nonribosomal genes has not yet been employed for identification or typing of carnobacteria.

Culture-independent genotypic methods offer the advantage that no caution has to be taken regarding the choice of bacteriological media for isolation. PCR-DGGE and PCR–temperature gradient gel electrophoresis have been used for detection of carnobacteria in meat and shrimp products, and offer an alternative to traditional culture-based methods (Ercolini et al. 2006;
Vasilopoulos et al. 2008; Brightwell et al. 2009; Jaffrès et al. 2009; Audenaert et al. 2010). However, comigrations of bands may hamper differentiation of *C. divergens* and *C. alterfunditum/C. pleistocenium* (Jaffrès et al. 2009), which underlines the necessity of validating results by sequencing. Real-time PCR has been proposed for rapid detection and quantification of *Carnobacterium* spp. from soft cheeses (Cailliez-Grimal et al. 2005).

References


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Chapter 7

Streptococcus: A Brief Update on the Current Taxonomic Status of the Genus

John R. Tagg, Philip A. Wescombe, and Jeremy P. Burton

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7.1 Historical Perspectives

“Streptococcus” derives from the Greek *streptos*—easily twisted like a chain—and *kokkos*—grain or seed—and the term was first used in 1874 by Billroth as a descriptor for the chain-forming, coccoid-shaped bacteria commonly detected in wounds and discharges from animal bodies and in about one-half of erysipelas cases (Billroth 1874). Later, Pasteur presented to the French Academy of Medicine on March 11, 1879, an account of his microscopic observations of streptococci isolated from the uterus and blood of women with puerperal sepsis and proposed an etiological association (Alouf and Horaud 1997). However, it was Rosenbach in 1884 who first applied the generic name *Streptococcus* when describing the chain-forming coccus isolated from suppurative lesions in man (Rosenbach 1884). He gave to this bacterium the species designation *Streptococcus pyogenes*.

In the ensuing 20 years the association between streptococci and a wide variety of diseases of man and other animals was firmly established, as was the importance of these bacteria in the dairy industry. The early attempts at classification focused on classical bacteriological tests such as assessment of host spectrum and pathogenicity, cultural appearances on agar or gelatin-based media, Gram stain appearance, reactions on blood agar and in milk cultures, and range of growth temperatures. The ability of some streptococci to produce hemolysis on blood agar became an important characteristic for identification, with the streptococci from pathological conditions in man almost invariably displaying complete (β) hemolysis (Schotmuller 1903).

In 1906 Andrewes and Horder examined 1200 streptococci isolated from human, air, and milk sources and on the basis of their sugar fermentation reactions, reduction of neutral red, and growth characteristics in milk distinguished eight groups (Andrewes and Horder 1906): *S. pyogenes*, as previously described by Rosenbach (1884); *Streptococcus equinus*, infrequently associated with diseases but commonly present in the intestinal tracts of herbivores and humans, and also commonly found in air and dust samples; *Streptococcus mitis*, only occasionally associated with diseases and mainly found in human saliva and intestinal contents; *Streptococcus salivarius*, predominantly isolated from human saliva and intestinal contents; *Streptococcus anginosus*, considered a long-chained pathogenic form of *S. salivarius* associated with sore throats and also found in the intestine; *Streptococcus faecalis*, isolated mainly from the human intestine and “pneumococci” distinguished by their capsule formation, but not at that time given a species designation within the genus *Streptococcus*.

Subsequently Orla-Jensen (1919) used an expanded range of tests including fermentation characteristics, tolerance to heat and sodium chloride, temperature limits of growth, and cellular morphology to help define nine groups of streptococci, mainly, however, comprising isolates from dairy sources (Orla-Jensen 1919).

It was Sherman (1937), however, who produced the first really comprehensive systematic classification of streptococcal isolates from a broad variety of sources; environmental, commensal, and disease associated. He excluded from the genus *Streptococcus* all strictly anaerobic cocci and also the pneumococci because of their extreme sensitivity to bile. Sherman delineated four primary divisions on the basis of their hemolytic activity, group carbohydrate antigens, strong reducing capability, ability to grow at 10°C and 45°C, ability to survive heating at 60°C for 30 min, and growth at pH 9.6 or in the presence of 0.1% methylene blue or in 6.5% sodium chloride. The divisions were termed “pyogenic,” “enterococcus,” “lactic,” and “viridans.” The pyogenic division comprised most of the species known then to be pathogenic for man and other animals. These were mostly β hemolytic and contained a group polysaccharide detectable by the Lancefield precipitin method (Lancefield 1933). Some (but not all) of the viridans cluster produced greening
(α hemolysis) on blood, hence the name viridans, from the Latin “viridis,” green. The name has subsequently often been misapplied as a general term or sometimes even as a species designation, “Streptococcus viridans.”

The “viridans” streptococci were commonly found as normal inhabitants of the mouth and throat, although some became invasive if the host resistance mechanisms were reduced (as with endocarditis). The designation “lactic” streptococci, although somewhat misleading since all streptococci produce lactic acid, was adopted for the species *Streptococcus lactis* and *Streptococcus cremoris*, since these common milk-souring streptococci had long been referred to as the “lactic acid streptococci.” They all expressed the Lancefield group N antigen. The members of the “enterococcus” division were distinguished by their wide range of growth temperatures and their tolerance to salt and alkali. Possession of Lancefield group D antigenicity was also a unifying feature. The physiological/biochemically based Sherman classification scheme, augmented by classical serological characterization was widely accepted for many years until superseded more recently by schema informed by the application of molecular methodologies.

The introduction of serological grouping following the seminal studies of Lancefield (1933) had a major and enduring influence on the classification of the hemolytic streptococci. Lancefield’s recognition of group-specific polysaccharides (the so-called C substances) that could be detected by precipitin reactions provided what at first appeared to be a direct correlation between certain of the serological groups (A to E and N) and particular *Streptococcus* species, as defined on the basis of physiological and biochemical tests (Lancefield 1933). Because of the relative ease with which serological grouping could be conducted using either acid (Lancefield 1933) or formamide (Fuller 1938) extracts of the streptococcal cells, β-hemolytic streptococcal isolates from clinical specimens were often identified as, for example, group A (*S. pyogenes*) or group B (*Streptococcus agalactiae*), and no further testing was done other than serological typing (Griffith 1934) for specialized epidemiological purposes. Because this worked so well (and indeed still does for these two species), a heavy dependence was placed on serological grouping for the routine identification of hemolytic streptococci. Subsequently many of the serological groups came to be regarded as homogeneous taxonomic entities (even as distinct species). Exceptions included group C, which was known to contain several species or biotypes; group D, which from the start was reported to comprise *S. faecalis* and *Streptococcus durans*; and group N, which contained both *S. lactis* and *S. cremoris*. With further study it became apparent that streptococcal isolates of quite unrelated species may harbor identical Lancefield antigens and strains that are genetically related at the species level may have heterogeneous Lancefield antigens.

More recently, the results of molecular taxonomic studies have led to the introduction of major changes in the classification of the streptococci; the “lactic” (Lancefield group N) streptococci now constitute the genus *Lactococcus* (Schleifer et al. 1985) and some of the streptococci from Sherman’s (1937) “enterococcus” division have become foundation members of the genus *Enterococcus* (Schleifer and Kilpper-Balz 1984; Sherman 1937). The motile streptococci have been transferred to the genus *Vagococcus* (Collins et al. 1989) and the anaerobic streptococci are now found within the *Peptostreptococcus* genus (Kluger and van Niel 1936). The so-called nutritionally variant streptococci were reasssembled within the *Abiotrophia* (Kawamura et al. 1995a) and then some of these were later displaced into the genus *Granulicatella* (Collins and Lawson 2000).

Although the traditional streptococcal phenotypic criteria (hemolytic reactions and Lancefield serology) are still widely used in the clinical setting for the indicative classification of streptococcal isolates, these cornerstones of streptococcal classification have now largely been superseded by contemporary molecular taxonomy principles.
7.2 Genus Characteristics and Species Discriminators

The members of the genus *Streptococcus* are gram-positive, catalase-negative, cytochrome-negative, facultatively anaerobic, spherical or ovoid bacteria, less than 2 µm in diameter and with a relatively low G+C content, ranging from 34 to 46 mol%. Taxonomically they belong to the family *Streptococcaceae* within the phylum *Firmicutes*, but more commonly they are regarded as members of the lactic acid bacteria. Division along a single axis causes streptococcal cells to grow as chains, particularly in liquid cultures, and some chains may contain 50 or more cellular units (Ekstedt and Stollerman 1960). Cross-walls form at right angles to the axis of the chain and after division, an appearance of more tightly conjoined cell pairs within the chain is sometimes evident. Short rod forms can occur, especially during growth on solid media. Streptococci are nonmotile, although some members of the species *Streptococcus sanguis* exhibit a curious “twitching motility” by using their type IV polar fimbriae in a grappling hook fashion to translocate across surfaces (Henriksen and Henrichsen 1975). Proteinaceous surface fibrils or filaments adorn the surface of many streptococci, sometimes arranged as tufts in some species. These fuzzy coats are known to have important roles in adhesion to surfaces and in protecting the streptococcus against phagocytosis. Most streptococci will grow in air, but some such as *Streptococcus pneumoniae* require additional carbon dioxide for satisfactory growth, a consequence of their adaptation to an oral cavity (carbon dioxide–enriched) habitat. All fail to reduce nitrate. They ferment glucose, predominantly with formation of lactic acid, but not gas.

7.3 Growth Characteristics *In Vitro*

Due to their complex nutritional requirements, the growth of most streptococci on basal nutrient media is relatively poor, but is aided by enrichment with blood, serum, or fermentable carbohydrate. Their colonies on agar are generally smaller (ca. 1 mm diameter after 24 h at 37°C) than those of staphylococci and unlike staphylococci, they are almost always nonpigmented, although exceptions under certain culture conditions include most *S. agalactiae* (orange–red) (de la Rosa et al. 1992) and many *Streptococcus mutans* (yellow) (Woltjes et al. 1982). The temperature optimum for most streptococci is around 37°C, although some species such as *Streptococcus uberis* and *Streptococcus thermophilus* grow at temperatures as low as 10°C or as high as 45°C, respectively.

Laboratory identification of some members of the so-called viridans and bovis groups of streptococci is facilitated by incorporation of sucrose into the growth medium (e.g., Mitis–Salivarius agar) (Chapman 1944). For example, the size of colonies of *S. salivarius* and of some *Streptococcus bovis* is augmented by the formation from sucrose of water-soluble extracellular levan (Niven et al. 1941), whereas colonies of the typical dental plaque species *S. sanguis*, *Streptococcus gordonii*, *Streptococcus oralis*, and most of the mutans streptococci are hard and cohesive due to their content of insoluble dextrans (MacFadden 1985).

Although the growth of streptococci in liquid cultures is enhanced by addition of fermentable carbohydrate, this increases lactic acid production, which in turn leads to more rapid cell death in the poststationary growth phase of the cultures. However, by buffering the media or through regular addition of alkali, heavy growth of most streptococci can be obtained in media such as Todd–Hewitt broth (Todd and Hewitt 1932).

* Truper and de’Clari (1997, 1998) proposed renaming several streptococcal species as follows: *S. sanguinis* to *S. sanguinis*, *S. parasanguinis* to *S. parasanguinis*, *S. crista* to *S. cristatus*, *S. rattus* to *S. ratti*, and *S. cricetus* to *S. criceti*. These changes, although grammatically correct, have been ruled by the Judicial Commission of the International Committee on Systematic Bacteriology (2000) to nevertheless be invalid (Kilian 2001).
The production by some streptococci of complete zones of hemolysis (β-hemolysis) when growing on the surface of blood agar has long been a key indicator for the presumptive detection of potentially pathogenic streptococci. Other streptococci either produce zones of greenish discoloration (α-hemolysis) or no discernable effect on the red blood cells (the latter perhaps somewhat illogically termed γ-hemolysis). Exceptions occur; some *S. pyogenes* are deficient in hemolysin production (Yoshino et al. 2010) and some members of commensal species such as *S. salivarius* exhibit β-hemolysis on certain blood agar media (Tompkins and Tagg 1987). The β-hemolysis of *S. pyogenes* strains is due to the activity of the well-defined hemolysins, streptolysin O and especially streptolysin S (Nizet 2002), whereas the α-hemolysis is attributed to hydrogen peroxide (formed during growth in the presence of oxygen by some streptococci) inducing the oxidation of the heme iron of hemoglobin with formation of methemoglobin (Barnard and Stinson 1996). This effect is more evident on chocolate agar, since the heating of the blood destroys the native catalase activity of the erythrocytes (Agar et al. 1986).

### 7.4 Nutritional Requirements and Metabolic Characteristics

The streptococci are in general nutritionally fastidious, indeed more so than their human host in their dependency on preformed amino acids and cofactors (Van der Rijn and Kessler 1980). In the laboratory their growth requirements are generally satisfied by blood agar media comprising a base of peptones, meat extract, and carbohydrate. Genome analyses show that *S. pyogenes*, *S. agalactiae*, and *S. pneumoniae* completely lack any tricarboxylic acid (TCA) cycle capability (Glaser et al. 2002), meaning they are unable to synthesize the precursors of most amino acids. On the other hand, some oral streptococci can grow in the presence of ammonia, sugar, vitamins, and salts. Indeed the *S. mutans* UA159 genome contains some amino acid biosynthetic pathways and a partial TCA cycle (Ajdic et al. 2002). In nature, the streptococcal requirement for nitrogenous compounds is satisfied by amino acids excreted by companion members of the oral microflora or released by bacterial proteinases acting on tissue proteins. The ability to utilize peptides is dependent on transport mechanisms in the cytoplasmic membrane and intracellular peptidases capable of hydrolyzing the peptides to the constituent amino acids. Oral streptococci have adopted various strategies to cope with environmental acidification, including adaptive acid tolerance responses and production of alkali. Ammonia-generating mechanisms used by some oral streptococci include the hydrolysis of urea by urease, especially by *S. salivarius* (Chen et al. 2000), and degradation of arginine using arginine deiminase by *Streptococcus rattus*, *S. gordonii*, and *S. sanguis*, a reaction that also yields energy (Griswold et al. 2004).

Streptococci are facultative anaerobes and most can flourish in the absence of oxygen. *S. pneumoniae* and many other oral species require elevated carbon dioxide levels for adequate growth and some other streptococci grow more favorably under anaerobic conditions. Their energy requirements are obtained from the fermentation of carbohydrates and indeed they are incapable of respiratory metabolism. Among the bacteria capable of growing aerobically, the streptococci are unique in that they are incapable of forming ATP via electron transport systems and they lack the ability to synthesize porphyrins, cytochromes, or catalase.

### 7.5 Classification

Serial revisions of the genus *Streptococcus* have been implemented since its genesis. The following subdivision into six major groups and one as yet ill-defined cluster of species is heavily based on 16S rRNA gene sequence data and correlates well with the results of DNA–DNA reassociation experiments and numerical taxonomy studies (Table 7.1) (Bentley et al. 1991; Facklam 2002;
### Table 7.1 Species and Subspecies of the Genus *Streptococcus*

<table>
<thead>
<tr>
<th>Phylogenetic Group</th>
<th>Species</th>
<th>Lancefield’s Group Antigen$^1$</th>
<th>Hemolysis$^2$</th>
<th>Main Habitat</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>Pyogenic group</td>
<td><em>S. pyogenes</em></td>
<td>A</td>
<td>$\beta$</td>
<td>Humans</td>
<td>Rosenbach (1884)</td>
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<tr>
<td></td>
<td><em>S. agalactiae</em></td>
<td>B</td>
<td>$\beta$ (α/–)</td>
<td>Humans, cattle</td>
<td>Nocard and Mollereau (1887)</td>
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<td></td>
<td><em>S. dysgalactiae</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>subsp. <em>equisimilis</em></td>
<td>C (A, G, L)</td>
<td>$\beta$</td>
<td>Humans</td>
<td>Vandamme et al. (1996)</td>
</tr>
<tr>
<td></td>
<td>subsp. <em>dysgalactiae</em></td>
<td>C (L)</td>
<td>$\alpha$ (β/–)</td>
<td>Pigs, cattle</td>
<td>Vandamme et al. (1996)</td>
</tr>
<tr>
<td></td>
<td><em>S. equi</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>subsp. <em>equi</em></td>
<td>C</td>
<td>$\beta$</td>
<td>Horses, donkeys</td>
<td>Farrow and Collins (1984)</td>
</tr>
<tr>
<td></td>
<td>subsp. <em>zooepidemicus</em></td>
<td>C</td>
<td>$\beta$</td>
<td>Many animals</td>
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</tr>
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<td></td>
<td>subsp. <em>ruminatorum</em></td>
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<td>$\beta$</td>
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<td></td>
<td><em>S. parauberis</em></td>
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<td>$\alpha/-$</td>
<td>Cattle</td>
<td>Williams and Collins (1990)</td>
</tr>
<tr>
<td></td>
<td><em>S. porcinus</em></td>
<td>(B), E, P, U, V</td>
<td>$\beta$</td>
<td>Pigs</td>
<td>Collins et al. (1984)</td>
</tr>
<tr>
<td></td>
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<td>–</td>
<td>$\beta$</td>
<td>Humans</td>
<td>Bekal et al. (2006)</td>
</tr>
<tr>
<td></td>
<td><em>S. canis</em></td>
<td>G</td>
<td>$\beta$</td>
<td>Many animals, humans</td>
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</tr>
<tr>
<td></td>
<td><em>S. hyointestinalis</em></td>
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<td>Incomplete $\beta$</td>
<td>Pigs</td>
<td>Devriese et al. (1986b)</td>
</tr>
<tr>
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<tr>
<td></td>
<td><em>S. thoraltensis</em></td>
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<td></td>
<td><em>S. halichoeri</em></td>
<td>B</td>
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<tr>
<td>Species</td>
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<td>β</td>
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<tr>
<td><em>S. castoreus</em></td>
<td>A</td>
<td>β</td>
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<td>β</td>
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<tr>
<td><em>S. iniae</em></td>
<td>−</td>
<td>β</td>
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<tr>
<td><em>S. urinalis</em></td>
<td>−</td>
<td>−</td>
<td>Humans</td>
<td>Collins et al. (2000)</td>
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<tr>
<td><em>S. plurianimalium</em></td>
<td></td>
<td>α</td>
<td>Cattle, goats, cats, canaries</td>
<td>Devriese et al. (1999)</td>
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Anginosus group ("milleri"-group)

<table>
<thead>
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<th>Species</th>
<th>−, F, C, A, G</th>
<th>β/α/−</th>
<th>Source</th>
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<table>
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<tr>
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<tbody>
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<td>β</td>
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Mitis group

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<th>Source</th>
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</thead>
<tbody>
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<td>−, K, O</td>
<td>α</td>
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</tr>
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<td><em>S. sanguis</em></td>
<td>−, H, W</td>
<td>α</td>
<td>Humans</td>
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<td>α</td>
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<td><em>S. pneumoniae</em></td>
<td>O</td>
<td>α</td>
<td>Humans</td>
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<tr>
<td><em>S. gordonii</em></td>
<td>−, H</td>
<td>α</td>
<td>Humans</td>
</tr>
<tr>
<td><em>S. crista</em></td>
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<td>α</td>
<td>Humans</td>
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<td><em>S. parasanguis</em></td>
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(continued)
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<tr>
<th>Phylogenetic Group</th>
<th>Species</th>
<th>Lancefield’s Group Antigen(^a)</th>
<th>Hemolysis(^a)</th>
<th>Main Habitat</th>
<th>Reference</th>
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<td></td>
<td><em>S. australis</em></td>
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<td>(\alpha)</td>
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<td><em>S. pseudopnemoniae</em></td>
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<td><em>S. peroris</em></td>
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<td>Humans</td>
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<td>(_), F</td>
<td>(\alpha)</td>
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<td></td>
<td><em>S. oligofermentans</em></td>
<td></td>
<td>(\alpha)</td>
<td>Humans</td>
<td>Tong et al. (2003)</td>
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<td></td>
<td>Humans</td>
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<td>(_) ((\alpha/\beta))</td>
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<td></td>
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<td>(\alpha)</td>
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<td><em>S. thermophilus</em></td>
<td>_</td>
<td>(_) /(\beta)</td>
<td>Milk, dairy products</td>
<td>Orla-Jensen (1919)</td>
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<td>Bovis group(^b)</td>
<td><em>S. bovis</em></td>
<td>(_), D</td>
<td>(\alpha/_)</td>
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<td>Orla-Jensen (1919)</td>
</tr>
<tr>
<td></td>
<td><em>S. equinus</em></td>
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<td>(\alpha)</td>
<td>Horses, other ruminants</td>
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<td>(\alpha)</td>
<td></td>
<td>Schlegel et al. (2003b)</td>
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<td></td>
<td>(subsp. <em>gallopyticus</em>)</td>
<td>(_), D</td>
<td>_</td>
<td>Marsupials, mammals incl. humans</td>
<td>Osawa et al. (1995)</td>
</tr>
<tr>
<td><strong>Species</strong></td>
<td><strong>Subspecies</strong></td>
<td><strong>Hosts</strong></td>
<td><strong>Reference</strong></td>
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</tr>
<tr>
<td><em>S. infantarius</em></td>
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<td>Humans</td>
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* A “–” represents no reaction with tested group sera or no hemolysis, while a blank indicates no information provided about Lancefield group or hemolysis.
* The bovis group is still undergoing significant taxonomic changes, some of which are indicated in this table.
* Schlegel et al. (2003b) has indicated that *S. bovis* and *S. equinus* should be combined within the priority species *S. equinus*.
* Schlegel et al. (2003b) subsumed *S. gallolyticus*, *S. macedonicus*, and *S. pasteurianus* as subspecies under *S. gallolyticus* although this has not to date been finally ratified.
Kawamura et al. 1995b; Kilian 2005; Kohler 2007). It should be noted that many of the more recently described species are as yet represented in the literature only by a very small number of independently isolated strains.

1. Pyogenic group—essentially β-hemolytic species pathogenic for humans and other animals
2. Anginosus group—commensal and occasional opportunistic pathogens found in the oral cavity and the gastrointestinal and genital tracts of humans
3. Mitis group—including the pathogen S. pneumoniae and various oral commensals
4. Salivarius group—comprises dairy streptococci and commensals of the human oral cavity
5. Bovis group—species found in the intestinal tract of several animal species
6. Mutans group—comprises genetically heterogeneous species that are nevertheless phenotypically similar
7. Currently ill-defined group—species not yet grouped or of uncertain phylogenetic relationships

### 7.5.1 Pyogenic Group

These streptococci are animal parasites and are typically capable of causing septicemia or respiratory tract infections. Mostly β hemolytic and displaying cell wall polysaccharide antigens of a variety of Lancefield group specificities, they are also commonly referred to as the hemolytic streptococci. Many of these species seem strongly adapted to particular animal hosts, although some also appear to be capable of causing zoonotic infection of close-contact humans. None is especially resistant to heat or grows at extremes of temperature, pH, or sodium chloride concentration, nor do they exhibit strong reducing capabilities.

By far the best-known member of this group is that versatile, enigmatic, and (almost) exclusively human pathogen, S. pyogenes. In the past S. pyogenes was the major cause of puerperal sepsis and scarlet fever. Now the most common cause of pharyngitis and impetigo in children, it can also cause a wide variety of deep and invasive infections—erysipelas, cellulitis, necrotizing fasciitis, and superantigen-induced streptococcal toxic shock syndrome (Mitchell 2003). Important post-infectious “nonpyogenic” syndromes include rheumatic fever and glomerulonephritis (Cunningham 2008) and also possibly the pediatric autoimmune neuropsychiatric disorders associated with streptococci (Shulman 2009).

In clinical practice the term group A streptococcus has been used almost synonymously for S. pyogenes. With the exception of some uncommon strains that express an A-variant carbohydrate (Elliott et al. 1971), all S. pyogenes appear serologically to be group A antigen positive. On the other hand, the converse is not true. Group A carbohydrate antigen reactivity has also been detected in strains of S. anginosus, Streptococcus dysgalactiae subspecies equisimilis, Streptococcus orisratti, and Streptococcus castoreus.

S. agalactiae, although long recognized as an important cause of mastitis in cattle, is also commonly resident in the human respiratory, genital, and gastrointestinal tracts and is an important pathogen of humans, especially of infants where it presents as either sepsis or meningitis (Berardi et al. 2007). Neonatal disease results from colonization of the maternal genital tract. Predisposing conditions for infection in nonpregnant adults include diabetes mellitus, cancer, and human immunodeficiency virus infection. As with S. pyogenes the species is well defined and serologically is consistently Lancefield group B, although strains from human sources differ from bovine strains in a number of pathogenic and phenotypic characteristics. Genotyping data have indicated that human and bovine-derived S. agalactiae represent mostly distinct populations, but recent studies
indicate some limited transmission may occur to humans exposed to cattle (Manning et al. 2010). *S. agalactiae* has also been identified as an emerging pathogen in aquaculture, associated with considerable morbidity and mortality in fish farms and moreover is sporadically associated with illness in various other mammalian and nonmammalian host species worldwide (Pereira et al. 2010). Two subspecies of *S. dysgalactiae* have been proposed as new taxa (Vandamme et al. 1996). *S. dysgalactiae* subspecies *equisimilis* includes isolates from humans and animals that show strong β hemolysis and typically express Lancefield group C or G antigens and occasionally group A or group L. They also express homologs of many *S. pyogenes* virulence genes, including those for many different M proteins. On the other hand, *S. dysgalactiae* subspecies *dysgalactiae* is isolated only from animals; shows α, β, or sometimes no hemolysis; and expresses either Lancefield group C or L antigenicity.

Three subspecies of the Lancefield group C *Streptococcus equi* are now recognized. *S. equi* subsp. *equi* causes “strangles” in horses and, to date, has not been isolated from humans. *S. equi* subsp. *zooepidemicus* is found in animal and human infections (Facklam 2002). *S. equi* subsp. *ruminatorum* has been recovered from milk samples from mastitis-affected goats and sheep (Fernandez et al. 2004).

The phenotypically closely related, but genetically distinctive species *S. uberis* and *Streptococcus parauberis* are economically important causative agents of bovine mastitis and in contrast to most other pyogenic streptococci they are α hemolytic or nonhemolytic. Some strains react with Lancefield group B, E, G, or P antisera (Groschup et al. 1991).

The species *Streptococcus porcinus* was proposed in 1984 to accommodate physiologically related β-hemolytic streptococci principally belonging to Lancefield groups E, P, U, and V (Collins et al. 1984). They are associated with pyogenic infections in swine and only rarely from other hosts, including human. More recently, several isolates from human genitourinary tract specimens were differentiated by 16S rRNA as the new species *Streptococcus pseudoporcinus* (Bekal et al. 2006).

*Streptococcus canis* are large-colony-forming group G streptococci isolated most commonly from dogs and mastitis-infected cows, but also sometimes causing infections in humans (Lam et al. 2007).

Relatively recent additions to the pyogenic cluster have been the swine-associated *Streptococcus hyointestinalis* (Devriese et al. 1986b), *Streptococcus hyovaginalis* and *Streptococcus thoraltensis* (Devriese et al. 1997), and the seal-associated *Streptococcus halichoeri* (Lawson et al. 2004) and *Streptococcus phocae* (Skaar et al. 1994). Other examples include one isolate of the Lancefield group A, *Streptococcus castoreus* from a beaver (Lawson et al. 2005b) and *Streptococcus didelphis* from opossums (Rurangirwa et al. 2000), the latter being of interest since it exhibits pseudo-catalase activity during its initial subculture on blood-free media. Also in this group are the emerging zoonotic pathogen *Streptococcus iniae* isolated from dolphins and fish in aquaculture and communicated to humans by contact with infected aquatic animals (Pier and Madin 1976); *Streptococcus urinalis*, which has been recovered from human urine (Collins et al. 2000); and *Streptococcus pluranimalium* from cattle and various other animal species (Devriese et al. 1999).

### 7.5.2 Anginosus Group

The anginosus group of streptococci are commensals of the oral cavity, gastrointestinal tract, and female urogenital tract that have substantial clinical relevance as agents of pharyngitis, pyogenic infections in various tissues and organs, and have a particularly strong association with abscess formation. The members include *S. anginosus, Streptococcus intermedius,* and *Streptococcus constellatus*—and within the latter, two subspecies are recognized, the subsp. *pharyngis* differing from subsp. *constellatus* in being Lancefield group C and chondroitin sulfatase positive (Grinwis et al.
The taxonomy of this group has become confused due to the lack of international consensus on their nomenclature (Facklam 1984). These streptococci have sometimes been referred to as the *Streptococcus milleri* group, although “*Streptococcus milleri*” has never been an officially approved name (Ruoff 1988).

The anginosus group of streptococci are small colony formers (<0.5 mm) and although some are β hemolytic, most are nonhemolytic or occasionally α hemolytic. Many isolates on blood agar produce a characteristic caramel odor that is due to diacetyl production (Chew and Smith 1992). Their identification is complicated by the diversity of phenotypes and antigenicity. Some strains carry Lancefield group antigen of either A, C, F, or G specificity (Facklam 2002).

### 7.5.3 Mitis Group

Many of the species in the mitis group have been considered difficult to classify and identify by biochemical methods due to the absence of reliable discriminatory traits. Members of the group are all closely related genetically and share high 16S rRNA gene sequence similarity. Some species are relatively poorly defined, and different species designations have sometimes been applied to the same strain. Core members of the group are *S. mitis*, *S. sanguis*, *S. oralis*, and *S. pneumoniae*.

*S. sanguis* initially referred to strains from subacute bacterial endocarditis, and two of its definitive phenotypic characteristics are IgA1 protease activity and extracellular glucan formation from sucrose. Some of the prototypical *S. sanguis*, such as strain Challis, have now been renamed *S. gordonii*, a species closely similar to *S. sanguis* but IgA1 protease negative (Kilian et al. 1989b). The mitis group species *Streptococcus crista* (resplendent in its characteristic tufted fibrils) (Handley et al. 1991) and also *Streptococcus parasanguis* and *Streptococcus australis* differ from *S. sanguis* in being IgA1 protease negative and glucan negative.

The group comprises many of the streptococcal species traditionally regarded as “viridans” (greening) streptococci, since they are predominantly α hemolytic. *S. parasanguis* is a cause of asymptomatic mastitis in sheep (Fernandez-Garayzabal et al. 1998). *S. orisratti* is unusual in that it has Lancefield group A antigenicity (Zhu et al. 2000). *Streptococcus lactarius* (Martin et al. 2010) was recently detected in breast milk samples from healthy women.

The versatile human pathogen *S. pneumoniae* (optochin and bile soluble) is placed within the mitis group on the basis of its phenotypic and genetic similarities to *S. mitis* and *S. oralis*. *S. pneumoniae* is an important agent of community-acquired pneumonia, sometimes accompanied by bacteremia. Other highly prevalent infections include otitis media, sinusitis, meningitis, and endocarditis (Mitchell and Mitchell 2010). *S. pneumoniae* typically colonizes the upper respiratory tract with no sign of infection (especially in children). Disease results from overactivation or dysregulation of the host inflammatory response (Henriques-Normark and Normark 2010). *Streptococcus pseudopneumoniae* was differentiated from *S. pneumoniae* following DNA–DNA hybridization studies and phenotypic tests (it is nonencapsulated and insoluble in bile) (Arbique et al. 2004).

### 7.5.4 Salivarius Group

The salivarius group comprises three species; *S. salivarius*, a pioneer colonizer of the human oral mucosa, mainly isolated from the tongue dorsum, the cheeks, and the palate; *Streptococcus vestibularis* (Whiley and Hardie 1988), a mutualistic bacterium present on the vestibulum of human oral mucosa; and *S. thermophilus*, a relatively thermophilic streptococcus used in the production of yogurt and Swiss- or Italian-type cooked cheeses (Schleifer et al. 1991). Recent phylogenetic analyses support a relatively close affiliation between *S. vestibularis* and *S. thermophilus* and an
early divergence of *S. salivarius* within the salivarius group lineage (Pombert et al. 2009). Whether *S. thermophilus* should be considered a subspecies of *S. salivarius* has been controversial, although DNA–DNA reassociation experiments indicated that they represent two separate species (Schleifer et al. 1991). The salivarius group is closely related to the bovis group and indeed *Streptococcus infantarius* and *Streptococcus alactolyticus*, which are now in the bovis group, were formerly placed in the salivarius group (Facklam 2002).

Most typically nonhemolytic on blood agar, *S. salivarius* produces distinctive mucoid colonies on sucrose-containing agar due to the production of water-soluble extracellular polysaccharides (levan). *S. salivarius* is finding increased application as a probiotic (Wescombe et al. 2009) and its excellent competitive capability within the oral ecosystem is attributed at least in part to its production of bacteriocin-like inhibitory substances (BLIS), most of which appear to be megaplasmid-encoded (Wescombe et al. 2006). *S. vestibularis* does not produce extracellular polysaccharide, but (along with many *S. salivarius*) is urease positive.

### 7.5.5 Bovis Group

The bovis group has experienced considerable taxonomic change in recent years, and the situation still appears to be in considerable flux. Phenotypic and genetic analyses of the type strains of “*S. bovis*” and *S. equinus* have indicated that the two species should be combined within the priority species *S. equinus* (Schlegel et al. 2003a). The isolates from human infections had previously been divided into *S. bovis* biotypes I, II/1, and II/2. The biotype I isolates, now reclassified as *Streptococcus galolyticus* subsp. *galolyticus* (Schlegel et al. 2003a), were most typically endocarditis associated and have been recognized to have an association with colon tumors since the late 1970s (Klein et al. 1977). Meanwhile the biotype II/2 isolates were reassigned as *Streptococcus galolyticus* subsp. *pasteurianus*, and the taxonomically synonymous *Streptococcus macedonicus* and *Streptococcus waius* were combined into *S. galolyticus* subsp. *macedonicus*. The less-frequently endocarditis-associated biotype II/1 strains formed the new species *S. infantarius* (Schlegel et al. 2000) and *Streptococcus lutetiensis* (Poyart et al. 2002). Another relatively well-separated member of the bovis group are the porcine and chicken isolates of *S. alactolyticus* (Farrow et al. 1984).

### 7.5.6 Mutans Group

The mutans streptococci are a cluster of relatively acidogenic and aciduric streptococcal species having similar characteristics, which include a propensity to bind to the tooth surface and a strong etiological association with the development of dental caries. The prototype species, named *S. mutans* by Clarke (1924) because of its dimorphic appearance in culture (cocci when growing optimally in liquid media and short rods on agar or in acidic liquid cultures), are mostly α hemolytic, although some β-hemolytic strains have also been described (Clarke 1924; Crooks et al. 1987). Genome analysis shows *S. mutans* can metabolize a wider range of carbohydrates than any other sequenced gram-positive bacterium (Ajdic et al. 2002).

*S. mutans* and *Streptococcus sobrinus* are the species most commonly isolated from human dental plaque. Although *Streptococcus cricetus* and *S. rattus* are occasionally isolated from humans, their primary hosts are hamsters and rats, respectively. Also isolated from rats has been *Streptococcus ferus*, whereas *Streptococcus macacae* and *Streptococcus downei* have been sourced solely from monkeys. More recent additions to the mutans group have included *Streptococcus dentirousetti* (Takada and Hirasawa 2008) and *Streptococcus orisuis* (Takada and Hirasawa 2007) isolated respectively from the oral cavities of bats and pigs and *Streptococcus devriesei* from equine teeth (Collins et al. 2004).
7.5.7 Ill-Defined Group

*Streptococcus suis* is an important pathogen of pigs worldwide and is also an emerging cause of zoonotic infection in the human contacts of infected animals (Kilpper-Balz and Schleifer 1987). Species relatively closely related to *S. suis* and also found associated with swine include *Streptococcus porci* (Vela et al. 2010), *Streptococcus plurextorum* (Vela et al. 2009), and *Streptococcus porcorum* (Vela et al. 2011). *S. alactolyticus* (and the phenotypically indistinguishable *Streptococcus intestinatis*) is another species to be isolated from the pig intestine (Vandamme et al. 1999).

Other relatively recently proposed species are *Streptococcus gallinaceus* from chickens (Collins et al. 2002), *Streptococcus ovis* from sheep (Collins et al. 2001), *Streptococcus ictaluri* from catfish (Shewmaker et al. 2007), *Streptococcus merionis* from Mongolian jirds (Tappe et al. 2009), *Streptococcus marimammalium* from seals (Lawson et al. 2005a), *Streptococcus difficile* (closely related to *S. agalactiae*) (Vandamme et al. 1997) from infected farmed tilapia and rainbow trout (Eldar et al. 1994), and *Streptococcus henryi* and *Streptococcus caballi* from equine sources (Milinovich et al. 2008).

*Streptococcus dentapri* (Takada et al. 2010) has been isolated from the oral cavity of wild boar and bats, while *Streptococcus entericus* (Vela et al. 2002) has been sourced from the cattle intestine.

7.6 Aggressors, Opportunists, and Protectors

Most of the streptococci are characterized as obligate parasites of mucosal (or sometimes tooth) surfaces of humans and other animals. Indeed, many are long-term, niche-adapted, predominant members of the commensal microflora of the upper respiratory, intestinal, or genital tracts of mammalian species. Exceptions, such as the dairy species *S. thermophilus*, are rare, especially since the reassignment in 1985 of the majority of the dairy streptococci to the genus *Lactococcus* (Schleifer et al. 1985). Most species have a preferred animal host, although some, especially *S. suis*, *S. equi* subsp. *zooepidemicus*, *S. canis*, *S. iniae*, *S. dysgalactiae* subsp. *equisimilis*, and possibly the bovine lineage of *S. agalactiae* can cause zoonotic infections.

Although some streptococcal commensals have no significant record of disease transgressions, others occasionally function as opportunistic pathogens, either if introduced into competitor-free (normally sterile) tissues or in immunologically compromised hosts. On the other hand, some streptococci are replete with virulence attributes and function as professional pathogens capable of interpersonal spread and of initiating infection in vulnerable (nonimmune protected) individuals.

Members of the disease-associated streptococcal species are known to also commonly occur in nondisease (carriage) relationships in which they are maintained in subdisease threshold numbers and thereby essentially simulate membership of the indigenous microbiota of the host. This feigned commensal-like behavior of the latent pathogens allows them to avoid the menacing attention of the host’s immunological defenses, and so for a time both the host and the streptococcus prosper. In some cases, oral streptococci can employ further subterfuge, entering buccal epithelial cells (LaPenta et al. 1994; Rudney et al. 2005), a state providing them with high level protection against immune (both innate and adaptive) and antibiotic (both therapeutic and bacteriocin) assault.

7.6.1 Aggressors

At one extreme of the parasitic streptococcal associates of humans are the sometimes aggressively pathogenic species *S. agalactiae*, *S. pneumoniae*, and *S. pyogenes*. The highest occurrence of serious disease associated with these species is found respectively in infants (meningitis and septicemia),
the elderly (pneumonia), and young adults (rheumatic carditis). On the other hand, in terms of total disease burden, the most prevalent streptococcal disease of humans is dental caries and the most highly incriminated etiological agent is \( S. \text{mutans} \).

The survival of individual streptococcal clones is ultimately dependent on their transmission to new hosts. The initial encounter of streptococcus and host takes place at epithelial surfaces, either the pharynx or skin. For \( S. \text{pyogenes} \) and \( S. \text{pneumoniae} \), this typically occurs either via infective droplets or by direct contact. For \( S. \text{agalactiae} \) and \( S. \text{mutans} \), mother-to-child transmission has particular significance—as a source of \( S. \text{agalactiae} \) infection for the neonate or of colonization with \( S. \text{mutans} \) following tooth eruption and especially during a “window of infectivity” period at around 2 years of age (Caufield et al. 1993).

The classic aggressive streptococcus \( S. \text{pyogenes} \) harbors a complex virulence repertoire of surface-associated and secreted components (Cunningham 2008; Mitchell 2003). Key elements of its pathogenicity are the hyaluronic acid capsules that mimic human tissue hyaluronic acid, proteases that specifically destroy the chemotactic signals that attract phagocytic defenses to infecting streptococci, and surface-bound M proteins that impede the phagocytic defenses and promote invasion of epithelial cells. Variations in M protein account for the more than 150 \( S. \text{pyogenes} \) serotypes.

### 7.6.2 Opportunists

For strains of the potentially pathogenic streptococcal species that are temporarily residing in subdisease threshold proportions within the indigenous microbiota of the human host, there are a number of changes either to the streptococcal population or to the animal host tissue environment that may effect a change in the relationship with the host to one that is regarded as disease.

- (a) Quorum sensing–enhanced upregulation of the expression of streptococcal virulence determinants
- (b) Decreased levels of natural competitors within the indigenous microbiota (e.g., following exposure of the host to antibiotics)
- (c) Development of defects in the host immune defenses (innate or induced)
- (d) Displacement of the potential pathogen to a relatively unprotected niche (e.g., in bacterial endocarditis)
- (e) Dietary changes (e.g., influence of sucrose supplementation on proliferation of \( S. \text{mutans} \) and initiation of dental caries)
- (f) Zoonotic transmission of a streptococcus from an alternative animal host
- (g) Acquisition of virulence determinants from other streptococci by horizontal gene transfer (mediated by transformation, transduction, or transmissible plasmids or transposons)

### 7.6.3 Protectors

Streptococci are predominant members of the commensal microbiotas of the mucous membranes of the human oral cavity and to a lesser extent of the nasopharynx. They are also transient members of the skin microbiota. The extent to which commensal streptococci colonize the exposed surfaces of other mammals is as yet unclear since the attention of researchers has to date largely been focused on the streptococcal aggressors. In humans, however, it is known that neonates typically acquire the mother’s predominant strain of the commensal species \( S. \text{salivarius} \) within days of birth (Tagg et al. 1983) and this raises the interesting prospect that the inheritance of at least certain components of the indigenous microbiota may predominantly be of a maternal lineage.
It was Pasteur who first introduced the notion of bacteriotherapy: the utilization of “harmless” bacteria to displace pathogenic organisms as a means of treating infection (Pasteur and Joubert 1877). Since commensal streptococci are particularly abundant in the human upper respiratory tract, they have been implicated as microbes having potential to interfere with colonization or infection by potentially pathogenic streptococci. Indeed, children who more frequently acquire S. pyogenes have been shown to have relatively fewer oral commensal streptococci exhibiting \textit{in vitro} inhibitory activity against S. pyogenes (Crowe et al. 1973; Holm and Grahn 1983). Such findings prompted Roos and co-workers (1993) to recommend dosing children with a mixture of \( \alpha \)-hemolytic streptococci as a supplementary treatment of streptococcal tonsillitis.

Of all the bacterial species known to populate the human oral and nasopharyngeal mucosa in large numbers, S. salivarius is perhaps the most innocuous (Burton et al. 2006). It has now been established that certain S. salivarius produce multiple BLIS, the activity of which is particularly strong against S. pyogenes. Children naturally harboring oral populations of S. salivarius producing the lantibiotics salivaricin A and/or salivaricin B were significantly less likely to acquire S. pyogenes (Dierksen and Tagg 2000). The prototype producer strain of these two BLIS activities, S. salivarius strain K12, is now widely used as an oral probiotic for the prevention and control of a variety of maladies, including halitosis, pharyngitis, and otitis media (Wescombe et al. 2009).

Another approach to the directed implementation of the principles of microbial interference focused on the application of a strongly competitive (bacteriocin-producing) strain of S. mutans that had its lactic-acid forming (cariogenic) capability disrupted by genetic modification. The modified strain is used to preemptively colonize and competitively exclude native (acidogenic) S. mutans from dental plaque as a strategy for prevention of dental caries (Hillman 2002). The same group has more recently developed a probiotic mixture of S. rattus, S. oralis, and S. uberis for use by humans and companion pets (Zahradnik et al. 2009).

Modulation of the microbiota composition by specific introduction of strains that are capable of excluding colonization/infection by target pathogens can be viewed as the controlled manipulation of a process that otherwise occurs only haphazardly in nature. It offers a cost-effective means of achieving protection for the host and it appears that bacterial replacement therapy will have an increasingly prominent role as a strategy for prevention and control of a wide variety of topical bacterial infections of humans and other animals.

From relatively humble beginnings, S. pyogenes, the twisted chain microbe of Billroth initially recognized as a common cause of pyogenic infection in humans and other animals, now finds itself positioned as the prototype of a rapidly expanding entourage of companion streptococcal species being isolated from both mammalian and nonmammalian hosts. The availability of increasingly sophisticated molecular methodologies to dissect the cellular content of complex microbiotas appears to have elicited an exponential increase in the discovery of novel streptococci. As researchers continue to probe ever more precisely within the commensal microbial populations of species in addition to \textit{Homo sapiens}, we can anticipate many more twists to the intriguing tale of the \textit{Streptococcus}.

References


Lactic Acid Bacteria: Microbiological and Functional Aspects


Chapter 8

Bifidobacteria: General Overview on Ecology, Taxonomy, and Genomics

Marco Ventura, Francesca Turroni, and Douwe van Sinderen

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8.1 General Features

Bifidobacteria were originally identified from stool samples of breast-fed infants in 1899 by Tissier, and termed Bacillus bifidus (Tissier 1900). Thereafter different designations have been employed such as Bacteroides bifidus and Lactobacillus bifidus. From 1973 bifidobacteria have been classified as a distinct genus (i.e., Bifidobacterium), originally including 11 species (Poupard et al. 1973). Later, Scardovi updated the number of bifidobacterial species to 24, which reached the latest number of 33 taxa (Ventura et al. 2007b), with the recent description of the new species Bifidobacterium bombi (Killer et al. 2009) and Bifidobacterium crudilactis (Delcenserie et al. 2007).
The main features that distinguish bifidobacteria from other bacterial groups focused on the fact that bifidobacteria are nonmotile, nonsporulating, catalase-negative, anaerobic, or microaerophilic, gram-positive, and with a high GC content (Ventura et al. 2007b). The name of the genus, Bifidobacterium, is derived from the fact that they typically appear as bifid, branched, or Y-shaped rods. However, under adverse growth conditions, their cells manifest high pleomorphism. It has been demonstrated that the presence of N-acetylglucosamine, alanine, aspartic acid, glutamic acid, serine, and Ca²⁺ ions in the growth medium influence the cell shape of bifidobacteria (Scardovi 1984).

8.2 Ecology of Bifidobacteria

The ecological origin together with genomic data represents an important starting point to understand the physiological and metabolic behavior of a bacterium. In fact, it is widely accepted that selection pressure is the driving force for ecological fitness that allows an organism to successfully survive and multiply in a particular environment. All bifidobacterial species described thus far are separated according to their ecological origin into six different ecological niches: the human intestine, oral cavity, food, the animal gastrointestinal tract (GIT), the insect intestine, and sewage. The “human” group of strains includes mainly those that were found in the intestine or stool samples of adults or infants and are represented by B. pseudocatenulatum, B. catenulatum, B. adolescentis, B. longum subsp. longum, B. longum subsp. infantis, B. breve, and B. angulatum (Lauer 1983; Scardovi 1974a, 1984). The presence of these bifidobacterial species in the human gut is often associated with the well-being of the host. Among bifidobacteria identified in the “human” group, it is worth mentioning two species, B. scardovii and B. dentium, which were originally isolated from blood and dental caries, respectively, suggesting a possible role of these microorganisms in the development of diseases. Many of the bifidobacterial species belonging to the “animal” group have been isolated from swine feces: B. longum subsp. suis, B. thermophilum, B. choerinum, B. aerophilum, B. psychroaerophilum, B. thermacidophilum subsp. porcinum, and B. boum (Matteuzzi et al. 1971; Mitsuoka 1969; Scardovi 1984; Simpson et al. 2003). Additional members of this group were obtained from feces of other mammals and birds (e.g., calf, cow, rabbit, and chicken) and include B. animalis subsp. animalis, B. magnum, B. pseudolongum subsp. pseudolongum, B. pseudolongum subsp. globosum, B. merycicum, B. ruminantium, B. saeculare, and B. cuniculi (Biavati and Mattarelli 1991; Lauer 1983; Scardovi 1974b, 1984). Four Bifidobacterium species have been isolated from the hindgut of the honeybee (B. asteroides, B. coryneforme, B. indicum, and B. bombi) (Killer et al. 2009; Lauer 1983), while three species have been isolated from sewage (B. minimum, B. subtilis, and B. thermacidophilum subsp. thermacidophilum) (Dong et al. 2000; Trovatelli et al. 1974), where it is likely that such species originate from fecal matter. Similarly, B. animalis subsp. lactis (Meile et al. 1997) was originally isolated from fermented milk, which raises the question as to whether this was its original environment or a contamination from another source.

A bifidobacterial survey from fecal samples collected from different animals (mammals as well as various species of birds) based on a PCR species-specific approach revealed an interesting bifidobacterial distribution, where only a small number of bifidobacterial species appear to be uniquely associated with some animals, while others display a much less restricted host range and consequently an extended ecological spread (Lamendella et al. 2008; Ventura et al. 2009b). Thus, species such as B. animalis were shown to be widely distributed within different animals’ GIT (i.e., possess a cosmopolitan lifestyle), whereas other taxa, such as B. gallinarum, B. thermophilum, B. longum, and B. breve, appear to possess a specialized lifestyle since they have exclusively been isolated from chickens, pigs, and human, respectively (Lamendella et al. 2008).
It is now widely accepted that there is also a specialization of bifidobacterial species in the human intestine according to an individual’s age. Thus, this explains the existence of an infant-specific bifidobacterial microbiota, which includes species such as *B. breve*, *B. bifidum*, and *B. longum* subsp. *infantis*, as well as an adult-specific bifidobacterial microbiota, represented by *B. adolescentis*, *B. catenulum/pseudocatenulum*, and *B. longum* subsp. *longum* species (Turroni et al. 2009a; Ventura et al. 2009b).

### 8.2.1 Human Intestinal Microbiota and Role of Bifidobacteria

The human large intestine is a very complex ecosystem, the microbial composition of which is yet to be fully determined, even if it is generally accepted that representatives of four bacterial phyla (*Bacteroidetes*, *Firmicutes*, *Actinobacteria*, and *Proteobacteria*) are the main residents of this ecosystem (Eckburg et al. 2005; for review see Turroni et al. 2008). The microbial population residing in the human intestine, which is named gut microbiota, represents one of the highest cellular densities in all known natural ecosystems (Eckburg et al. 2005). Notably, and in contrast to other ecosystems, the microbial biodiversity found in the human gut is relatively low (Turroni et al. 2008), where the dominant bacterial taxa are restricted to *Bacteroidetes* and *Firmicutes* (Eckburg et al. 2005; Turroni et al. 2008). Bacterial colonization of the human gut occurs immediately upon delivery and is influenced by several factors such as infant diet (e.g., breast feeding or formula feeding) and hygiene conditions (Fanaro et al. 2003). One of the first microbial colonizers is represented by the bifidobacteria, whose numbers appear to decline with age (Claesson et al. 2010; Turroni et al. 2008). Thus far, very little is known about the absolute number of the indigenous human intestinal bifidobacteria. In fact, the majority of the studies thus far performed are based on the analysis of fecal samples as an assumed valid representation of the microbial gut composition. However, fecal environments are colonized by members of not only the human gut microbiota, that is, mucosa-adherent or indigenous or autochthonous bacterial component, but also of bacteria that reside in the lumen, also referred to as transient, contaminant, or allochthonous microbiota (Sonnenburg et al. 2006). The latter elements of the microbiota are presumed to have originated from the diet and are just temporally present in the lumen of the intestine to end up in fecal material. Therefore, investigation of the microbiota present in fecal material is not necessarily reflecting the extent of bacterial biodiversity that is in direct contact with the intestinal mucosa. Recently, a considerable bifidobacterial ecological survey involving colonoscopic biopsies from healthy human patients was carried out (Turroni et al. 2009a). Such analyses were based on a polyphasic approach involving isolation of bifidobacteria using selective media together with molecular analysis of the rRNA gene sequences (16S rRNA gene and internally transcribed spacer 16S–23S spacer sequences). The culturable bifidobacterial population from intestinal samples was shown in this study to encompass members of six main phylogenetic taxa (*B. longum*, *B. pseudocatenulatum*, *B. adolescentis*, *B. pseudolongum*, *B. breve*, and *B. bifidum*) and two species mostly detected in fecal samples (*B. dentium* and *B. animalis* subsp. *lactis*). The latter finding suggests that these two species do not belong to the human intestinal mucosa-adherent bifidobacterial population. Furthermore, this study highlighted a considerable intersubject variability. Conversely, a very limited diversification of bifidobacterial populations was noticed between different intestinal regions within the same individual (intrasubject variability) (Turroni et al. 2009a).

Metagenomic and microbiomic analyses represent novel culture-based approaches used to investigate the microbial diversity of the human gut populations of the intestinal microbiota (Eckburg et al. 2005; Gill et al. 2006; Kurokawa et al. 2007; Palmer et al. 2007; Wang et al. 2005). However, a limited number of bifidobacterial rRNA gene sequences have thus far been detected...
in the above-mentioned metagenomic studies. It is worth mentioning that the data collected in these ecological surveys may have been skewed by the efficacy of the protocols used for extracting DNA directly from the environmental samples (e.g., fecal), as well as by the accuracy of PCR primers and the bias of the applied PCR conditions. Thus, it could be argued that the low number of detected bifidobacterial phylotypes may be due to poor cell lysis, which is a known problem when isolating DNA from bifidobacteria or indeed other Actinobacteria (Kauffmann et al. 2004; Turroni et al. 2008). A recent analysis of the human mucosa–adherent bifidobacterial population based on 16S rRNA sequences highlighted the existence of novel bifidobacterial rRNA sequences (Turroni et al. 2009b), indicating that these might represent novel bifidobacterial species (Turroni et al. 2009b). Furthermore, a bifidobacteria intestinal survey using a genomic DNA-based microarray technology allowed the identification of novel unknown phylotypes (Boesten et al. 2009).

8.3 Genomics and Bifidobacteria

Access to completely decoded microbial genomes is a crucial prerequisite to reconstruct events of genome evolution and metabolic capabilities. Evolutionary genomics, also known as phylogenomics, is concerned with phylogenetic analysis based on genes that are conserved among different bacteria (and therefore constitute the so-called core genome). Efforts have also been placed on the investigation of the bacterial pan-genome, representing the core genome plus the variable elements of a genome. Such variable elements include genes that are absent from the genome of at least one member of a taxonomic unit or genes that are unique to a single member of that taxonomic unit (Bentley 2009; Lapierre and Gogarten 2009; Rasko et al. 2008; Tettelin et al. 2005).

In 2002, the first bifidobacterial genome, that of *B. longum* subsp. *longum* NCC2705 (Schell et al. 2002), was made publicly available. Since then the genomic era has become prominent for the *Bifidobacterium* genus, with the generation of an additional nine complete bifidobacterial genomes (Table 8.1) (Barrangou et al. 2009; Kim et al. 2009; Lee et al. 2008; Sela et al. 2008; Ventura et al. 2009d). Furthermore, partly assembled genomes of 18 other bifidobacterial strains/species (Table 8.1) have become available in the National Center for Biotechnology Information database. Some of these genomes are derived from different strains within the same species (i.e., *B. longum*, *B. animalis* subsp. *lactis*, *B. adolescentis*, and *B. dentium*). Comparative studies using these genome sequences displayed a pan-genome structure consisting of more than 5000 genes, many of which are uncharacterized but probably crucial for the provision of adaptive capabilities pertinent to the human GIT (Bottacini et al. 2010). The pan-genome analysis also allowed the identification of truly unique genes (TUG), that is, genes present only in a reference genome but absent in any of the other available bifidobacterial genomes. Predicted functions of such TUGs are adhesion-mediating proteins (e.g., fimbrial subunits) and sugar-metabolizing enzymes (Bottacini et al. 2010). Recently, a new scientific concept called probiogenomics was proposed (Ventura et al. 2009a), which aims to provide insights into the diversity and evolution of probiotic bacteria, including probiotic bifidobacteria, and to reveal the molecular basis for their health-promoting activities. Furthermore, probiogenomics aims to uncover how probiotic bacteria, including bifidobacteria, sense and adapt to the GIT (Ventura et al. 2009a).

8.3.1 Biosynthetic Capabilities

Genomics data are extremely useful in reconstructing the metabolic capabilities of the organism under investigation. Furthermore, analysis of genomic information is important to facilitate the
Table 8.1 Bifidobacterial Genome Sequencing Projects

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</table>
development of new or improved methods for growth and isolation. In the case of bifidobacteria, it is well known that these microorganisms are difficult to cultivate in defined media, apparently due to their highly exigent nutrient demands.

On the basis of available bifidobacterial genome information, it is deduced that bifidobacteria have the genetic capability to synthesize at least 19 amino acids from ammonium and major biosynthetic precursors (e.g., pyruvate, fumarate, oxaloacetate, and oxoglutarate) supplied by the bifid shunt and by the partially present tricarboxylate acid cycle, which lacks fumarase, oxoglutarate dehydrogenase, and malate dehydrogenase (Schell et al. 2002). Whether and how bifidobacteria can synthesize cysteine is not fully clear, as genes for the sulfate/sulfite assimilation pathway appear to be absent in \textit{B. longum} subsp. longum NCC2705 (Schell et al. 2002) and other characterized bifidobacteria. However, enteric bifidobacteria may be able to synthesize cysteine using the homologs of cysteine synthase/cystathionine β synthase, \textit{O}-acetylhomoserine aminocarboxypropyltransferase, and cystathionine synthase starting from a reduced sulfur source (e.g., hydrogen sulfide), which may be present through the action of sulfur-reducing colonic microbiota, suggesting a synergistic relationship. With respect to the metabolic capabilities of bifidobacteria to synthesize pyrimidines and purine nucleotides, analysis of various bifidobacterial genomes has indicated that such microorganisms possess all the homologs needed for their \textit{de novo} production from glutamine. The genomes of \textit{B. longum} subsp. longum NCC2705, \textit{B. longum} subsp. longum DJ010A, \textit{B. longum} subsp. \textit{infantis} ATCC15697, and \textit{B. dentium} Bd1 are predicted to encode all the necessary enzymes for the synthesis of folic acid, nicotinate, and thiamin (Lee et al. 2008; Schell et al. 2002; Sela et al. 2008; Ventura et al. 2009d). In contrast, pathways needed for the \textit{de novo} biosynthesis of biotin, cobalamin, pantothenate, lipoate, and pyridoxine appear to be partially or completely absent (Lee et al. 2008; Schell et al. 2002; Sela et al. 2008; Ventura et al. 2009d). Furthermore, the biosynthetic pathway required for riboflavin seems absent in \textit{B. longum} subsp. longum but present in \textit{B. longum} subsp. \textit{infantis} (Lee et al. 2008; Schell et al. 2002; Sela et al. 2008), which indicates that this property is dependent on the subspecies or even the strain.

These biosynthetic capabilities indicate that \textit{Bifidobacterium} species have adapted to an environment where they cannot rely on an extraneous source of amino acids, nucleotides, and certain vitamins. Preliminary comparative genome studies complemented by the development of a defined synthetic medium for bifidobacteria have confirmed many of these predicted prototrophic and auxotrophic characteristics (O’Connell-Motherway, Forde, Fitzgerald, and van Sinderen, unpublished results).

Notably, the gene contents of bifidobacterial genomes reflect their adaptation to the human GIT. In this context, the chromosomes of enteric bifidobacteria such as \textit{B. longum} subsp. longum NCC2705 or \textit{B. longum} subsp. longum DJ010A contains a large arsenal of genes involved in the breakdown of complex diet- and host-derived carbohydrates (see paragraph below) that escape digestion from the proximal regions of the GIT. In contrast, the genome sequences of the oral-adapted \textit{B. dentium} Bd1 contains a large variety of genes dedicated to the metabolism of human saliva-derived compounds, acid tolerance, defense against antimicrobials, and to the metabolism of a vast array of sugars, including simple and complex carbohydrates (Ventura et al. 2009d). In particular, the high level of inherent tolerance of \textit{B. dentium} Bd1 to acidification have been related to the presence of a membrane-bound, proton-translocating FìFò ATPase system as well as of the genetic locus encoding for a glutamate decarboxylase (\textit{gadB-gadC}) (Ventura et al. 2009d).

### 8.3.2 Metabolic Capabilities

Genome sequencing represents the gold standard for the analyses of the full genetic blueprint of an organism, allowing the prediction of the genetic basis sustaining the metabolic capabilities...
of an organism. Such information is crucial for the understanding of the genetic adaptability of an organism to the specific corresponding ecological niche. A representative example for the latter consideration is offered by the microbial metabolism of dietary sugars, which appear to have a major impact on the genome contents of the human microbiome along the GIT. The human genome does not appear to encode enzymes that are capable of hydrolyzing and metabolizing complex carbohydrates, such as those of plant or animal origin (fructo-oligosaccharides, galacto-oligosaccharides, xylo-oligosaccharides, lactulose, and raffinose) (Guarner and Malagelada 2003), which constitute a normal component of the human diet. These dietary compounds would have been lost if the gut of human beings had not been colonized by a diverse microbiota, members of which include, among others, Bacteroides, Bifidobacterium, Clostridium, and Enterobacterium (Eckburg et al. 2005), which display the metabolic capacity to degrade such oligo- and polysaccharides. The energy needs of these bacteria are provided by the fermentation of these compounds that have been left undigested in the small intestine (Cummings and Englyst 1987; Delzenne and Williams 2002; Sinha and Kumria 2001).

In addition, the fermentation products of these substrates can be used by the host and in some cases have been shown to possess additional beneficial properties, including immune modulation, and anticarcinogenic and antiobesity activities (Belury 2002). In such a mutualistic interaction, enteric microorganisms are provided with a rich pabulum of carbohydrates in a relatively constant environment, whereas the host is given access to large amounts of short-chain fatty acids for energy and other possible health benefits. The capacity to degrade complex sugars, with respect to the degree of polymerization and type of glycosidic bonds, contributes to the competitiveness of a given microorganism in the GIT (Turrioni et al. 2008). In this context, simple sugars are metabolized rapidly in the proximal regions of the GIT, whereas complex sugars remain abundant in the lower parts of the GIT (e.g., the human colon). It makes sense for common inhabitants of the mammalian colon, such as bifidobacterial species, to dedicate a significant proportion of its coding capacity to carbohydrate metabolism (breakdown and transport), reflecting a critical adaptation to this highly competitive ecological niche.

Genomes of bifidobacteria possess a high proportion of predicted proteins (about 10% of their predicted proteome) that belong to the carbohydrate transport–metabolism family of clustered ortholog genes (Ventura et al. 2007b). The predicted enzymatic arsenal encoded by a microorganism for the degradation and utilization of carbohydrates as deduced from its genome content encompasses a variety of glycosyl hydrolases, glycosyl transferases, and glycosyl lyases (Coutinho and Henrissat 1999). Analyses of bifidobacterial genomes revealed an extensive but variable set of glycosyl hydrolases and glycosyl transferases, with the largest one detected in the oral inhabitant B. dentium Bd1 (Ventura et al. 2009d). In contrast, intestinal bifidobacterial genomes (e.g., B. longum subsp. longum NCC2705, B. longum subsp. longum DOJ10A, B. longum subsp. infantis ATCC15697, and B. adolescentis ATCC15703) possess a smaller repertoire of glycosyl hydrolases, glycosyl transferases, and glycosyl lyases (Lee et al. 2008; Schell et al. 2002; Sela et al. 2008). This indicates that these bacteria show a differential potential with respect to sugar utilization, perhaps linked to the different ecological niche occupied (e.g., human mouth vs. human colon). Furthermore, several of these glycosyl hydrolases are assumed to exert their activity outside the cytoplasm as secreted enzymes.

Another important aspect of carbohydrate metabolism is represented by the internalization of these molecules, which is mainly carried out through the action of ATP-binding cassette (ABC) transporters, permeases, and proton symporters, rather than through phosphoenolpyruvate phosphotransferase systems (Maze et al. 2007). Moreover, genome analyses of bifidobacterial genomes allowed the identification of genes or operons specifically involved in the breakdown of complex
polysaccharides, such as starch, amylopectin, glycogen, and pullulan (O’Connell Motherway et al. 2008; Ryan et al. 2006). Arabinofuranosyl-containing oligosaccharides obtained from plant cell wall polysaccharides arabinan, arabinogalactan, and arabinoxylan can be fermented by bifidobacteria (Van Laere et al. 2000). However, fermentation studies of bifidobacteria with arabinoxylan and arabinogalactan showed that the rate of degradation of these polymers is rather low compared with the oligosaccharides that can be derived from this polymer (van den Broek et al. 2008). A small number of bifidobacterial species, for example, *B. longum* subsp. *infantis* ATCC15697 and *B. bifidum* JCM1254, are able to utilize isomeric lacto-N-tetraose (Gal(β1-3)GlcNac(β1-3)Gal(β1-4) Glc) (Sela et al. 2008; Wada et al. 2008), which constitutes the core of human milk oligosaccharides (HMOs). Such sugars are metabolized through the activity of a lacto-N-biose phosphorylase and associated genes (Nishimoto and Kitaoka 2007). Additional enzymes predicted to metabolize HMOs and derivatives (e.g., sialic acid) are encoded by the genome of *B. longum* subsp. *infantis* ATCC15697. Notably, in the chromosome of this microorganism, a 43-kb gene cluster was identified including a large set of genes predicted to be involved in the metabolism of HMOs, for example, genes encoding fucosidase, sialidase, β-galactosidase, and β-hexosaminidase, which are associated with genes that are predicted to specify ABC transporters specific for such HMOs.

### 8.3.3 Bifidobacteria and Host Interactions

Extracytoplasmatic proteins and extracellular structures (e.g., capsular polysaccharides) have important roles as mediators of the molecular interactions between a bacterium and its environment. In the case of bifidobacteria, these structures mediate key functions affecting the host, such as adhesion, nutrient availability, immune system modulation, or pathogen inhibition/exclusion. Interesting cell surface–encoding proteins are fimbriae-like structures, which were identified owing to the presence of their encoding genes on the genome of enteric (Schell et al. 2002) as well as oral bifidobacteria (Ventura et al. 2009d). Although the exact role of such structures in bifidobacteria have not yet demonstrated, in other human GIT inhabitants, such as *Lactobacillus rhamnosus* GG, they have been shown to mediate microbial adhesion to and colonization of epithelial mucosal, or other host cell surfaces (Kankainen et al. 2009). Furthermore, the genomes belonging to certain bifidobacterial species (e.g., *B. breve*, *B. longum* subsp. *longum*, *B. longum* subsp. *infantis*, and *B. dentium*) have been shown to contain a gene whose predicted product shares similarity with serine-like protease inhibitor (serpin) proteins found in other bacteria and eukaryotes (Ivanov et al. 2006; Turrioni et al. 2010). Members of the serpin family regulate various signaling pathways in eukaryotes, and some are known for their ability to suppress inflammatory responses by inhibiting elastase activity (Potempa et al. 1994). Recent findings have demonstrated that the bifidobacterial serpin-like protein performs an immunomodulatory role in a murine colitis model by reducing intestinal inflammation (Ivanov et al. 2006).

Another mode of bifidobacteria–host interaction is represented by cell surface–decorating sugar structures, such as exopolysaccharides (EPS). The role of EPS produced by nonpathogenic colonic bacteria is not completely defined. However, it is plausible to suggest that these extracellular structures are important for microorganisms to establish themselves within the host (Ruas-Madiedo et al. 2006). Interestingly, the human intestinal microorganism *Bacteroides thetaiotaomicron* has developed a genetic mechanism allowing it to modify its surface makeup by synthesizing at least eight distinct EPS, which may provide the microorganism with opportunities to escape recognition by the host’s immune system (Krinos et al. 2001). In bifidobacteria, various reports have described the presence of such extracellular structures (Habu et al. 1987; Nagaoka et al. 1995, 1996; Ruas-Madiedo et al. 2007, 2009; Salazar et al. 2008). The genome of *B. longum*...
subsp. longum NCC2705 contains two regions related to EPS biosynthesis, which are flanked by insertion sequence (IS) elements and show a strong divergence in G+C content relative to the remainder of the genome, suggesting that such regions have been acquired by horizontal gene transfer. Similarly, analyses targeting the genomes of oral bifidobacteria such as B. dentium Bd1 revealed the presence of DNA regions that encode enzymes required for the synthesis of strain-specific EPS. However, analysis of the thus far complete bifidobacterial genomes indicates that not all bifidobacterial genomes contain such genetic clusters (Ventura et al. 2007b).

The molecular impact of bifidobacteria (e.g., B. longum) on the human host has recently been investigated (Sonnenburg et al. 2006). Notably, the host response to B. longum subsp. longum NCC2705 colonization involves the activation of T-cell-produced cytokine interferon and reduced host production of antibacterial proteins such as regenerating islet-derived-3 and pancreatitis-associated proteins (Sonnenburg et al. 2006).

### 8.3.4 Role of Genomics on Bifidobacterial Taxonomy

Genomics has provoked considerable advances on prokaryotic taxonomy through the delivery of a large dataset of gene sequences that can be used to infer bacterial phylogeny. Bacterial species are currently characterized by a polyphasic approach, which takes into consideration both genotypic and phenotypic (including chemotaxonomic) features (Stackebrandt et al. 1997; Vandamme et al. 1996). Since the 1970s, DNA–DNA hybridization methods have been considered the cornerstone of bacterial taxonomy, and at present a bacterial species is “a category that circumscribes a genomically coherent group of individual isolates/strains sharing many unconnected features, comparatively tested under highly standardized conditions” (Coenye et al. 2005). Thus, the genetic similarity between microorganisms is evaluated by the level of genome hybridization under well-defined conditions. Bacteria showing a DNA–DNA hybridization value higher than 70%, while displaying less than 5% difference in their melting temperature, are considered to belong to the same species. In contrast, bacterial isolates sharing a level of genomic DNA hybridization that is less than 50% do not belong to the same species.

The classical gene used in bacterial taxonomy is the 16S rRNA gene (Vandamme et al. 1996). Strains depicting more than 97% of 16S rRNA sequence identity are considered to belong to the same species, which is in most cases consistent with a minimal genomic hybridization level of 70%.

Owing to the introduction of comparative sequence analysis of the 16S rRNA gene, bifidobacterial taxonomy has greatly advanced (Matsuki et al. 1998, 1999; Ventura et al. 2001a, 2001b, 2003b; Ventura and Zink 2002). Moreover, in recent years, other molecular markers, including housekeeping genes such as recA (Kullen et al. 1997; Ventura and Zink 2003), tufA (Ventura et al. 2003a), atpD (Ventura et al. 2004a), groEL (Jian et al. 2001; Ventura et al. 2004b), dnaK (Ventura et al. 2005d), grpE (Ventura et al. 2005d), clpP (Ventura et al. 2005c), and hrcA (Ventura et al. 2005a), have been applied to bifidobacterial taxonomy. However, several studies raised concerns that phylogenetic analyses carried out on a single gene may not adequately reconstruct the real extent of evolutionary development of a microorganism (for review see Ventura et al. 2007a). More recently, phylogenetic analyses based on a set of combined alignments of conserved orthologous proteins (the so-called supertrees) (Bininda-Emonds 2004) has been introduced and offer a most reliable picture of evolutionary relationships between bacteria (Brown et al. 2001).

In the case of bifidobacteria when such a multigene-based approach was applied, six different phylogenetic clusters (i.e., B. longum, B. adolescentis, B. pullorum, B. pseudolongum, B. boum, and B. asteroides) were distinguished (Ventura et al. 2006). Interestingly, such a phylogenetic analysis of the genus Bifidobacterium based on the concatenation of six gene sequences (i.e., clpC, xfp, dnaJ1, rpoC, dnaB,
dnaG, and purF) revealed the increase in discriminatory power and robustness of the derived phylogenetic tree (Ventura et al. 2006). Moreover, this analysis showed that the projected ancestor of all recognized bifidobacteria groups was most closely related to the current B. asteroides species.

### 8.3.5 Mobile Elements in Bifidobacterial Genomes

Genomic analyses have indicated that mobile elements contribute significantly to bifidobacterial genome structure, with the presence of variable numbers of IS, (episomal) plasmids, and prophage-like elements (Barrangou et al. 2009; Kim et al. 2009; Lee et al. 2008; Schell et al. 2002; Sela et al. 2008; Ventura et al. 2009d). Eight IS families have been identified in bifidobacterial genomes sequenced thus far, including IS3, IS21, IS30, IS110, IS150, IS256, IS607/IS200, and ISL3. The largest arsenal of IS elements have been identified in the genome sequences of B. longum subsp. infantis ATCC15697, whereas the smallest IS repertoire was detected in the chromosome of B. dentium Bd1. IS elements are often associated with genome rearrangement or deletion events (Darling et al. 2008), and are therefore major driving forces in the acquisition or loss of important functions. In the case of B. longum subsp. longum DJO10A, lantibiotic production is associated with a gene flanked on both sides by IS elements (Lee et al. 2008), whose stability is affected by prolonged cultivation of this strain on synthetic media.

Another mobile element identified specifically in the genomes of B. longum subsp. longum is represented by a novel mobile integrase cassette (MIC) structures consisting of three contiguous integrases flanked by an inverted repeat and a palindrome structure formed by two IS3-type IS elements (Lee et al. 2008). In the genome of B. longum subsp. longum DJO10A, such a mobile element was found to be deleted together with a 50-kb genome sequence upon prolonged pure culture adaptation, thus reinforcing the role of MIC as a mobile element of bifidobacteria.

Among mobile genetic elements in bifidobacterial genomes, the presence of prophage-like elements is in particular remarkable since they constitute about 3% of the pan-genome sequences of the genus Bifidobacterium (Ventura et al. 2005b, 2009c). Genome analyses of bifidobacteria allowed the identification of 19 prophage-like elements showing high sequence identity to genes of double-stranded DNA bacteriophages spanning a broad phylogenetic range of host bacteria (Actinobacteria and Firmicutes) (Ventura et al. 2009c). Interestingly, even if most of the prophage-like elements identified in bifidobacterial genomes appear to be defective prophages, a small number of these prophage-like elements (i.e., Bdent-2 from B. dentium Bd1, Binf-4 from B. longum subsp. infantis ATCC15697, Blj-1 from B. longum subsp. longum DJO10A, and Ban-1 from B. animalis subsp. lactis ADO11) were shown to be inducible (Ventura et al. 2005b, 2009c), constituting an exciting opportunity for further studies on the biology of these so-called bifidophages.

### 8.4 Conclusions

The genomics era has recently embraced members of the genus Bifidobacterium, providing a significantly better understanding of the evolutionary path followed by this group of bacteria. Furthermore, genomic and comparative genomic analyses of bifidobacteria have highlighted key genes of these microorganisms, such as genes involved in carbohydrate metabolism, which are worthy of continued investigation for their potential roles in colonization of the human gut. In this context, ecological surveys based on new culture-independent techniques coupled with the understanding of the metabolic capabilities and nutrient requirements of bifidobacteria will provide the identification of novel bifidobacterial species from the human gut. A final issue is
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constituted by the future availability of all the genome sequences of the 33 species currently recognized of the *Bifidobacterium* genus. Such achievement will be crucial to provide a more robust image of phylogenetic relationships that exist between the different bifidobacterial species as well as how the genus *Bifidobacterium* has developed as an independent taxonomic unit within the Eubacteria. Taken in a broader context, it can be expected that the availability of a larger number of sequenced genomes will facilitate the development of universal genome sequence analysis schemes, which will allow the adoption of a more natural species concept.

Commensals such as bifidobacteria must coexist with their host and must evade or survive the diversity of responses that the host has generated to contrast and eradicate unwanted and pathogenic bacteria. Understanding the molecular mechanisms underlying the fascinating ability of the human immune system to differentiate between beneficial and harmful bacteria is a significant scientific challenge for the future.

Despite the advanced insights into microbial composition, bifidobacterial activity and bifidobacteria–host interactions in the GIT, these new findings also underline our limited understanding of the processes ongoing in this environmental niche.

References


Chapter 9

Bacteriophage and Anti-Phage Mechanisms in Lactic Acid Bacteria

Susan Mills, R. Paul Ross, Horst Neve, and Aidan Coffey

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9.1 Introduction

Bacteriophages (phages) are the principal cause of starter culture failure in many food fermentations carried out by lactic acid bacteria (LAB). These bacterial viruses were first characterized in the early 20th century by the work of Felix d’Hérelle in 1917, although their inhibitory activity had previously been documented by Ernest Hankin in 1896, Nikolay Gamaleya in 1898, and Frederick Twort in 1915 (O’Flaherty et al. 2009). Phage morphology remained undetermined until electron microscopy became available during the 1940s. Nevertheless, phages attacking Lactococcus lactis starter cultures have been known since the 1930s, when Whitehead and Cox
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in New Zealand observed that they were responsible for failed lactic acid development in cheese production (Whitehead and Cox 1935). In the area of food fermentations, the permanent threat of phage contamination is particularly manifested in the dairy field. Here phage infections of LAB during the fermentation process result in an unacceptably low production rate of lactic acid and flavor compounds along with reduced proteolysis. Thus, starter activity is either severely affected (“slow vats”), or in extreme cases, a complete failure of starter growth may occur (“dead vats”). Due to resulting financial losses for the dairy fermentation industry, control of phages is an area of concern in handling starter cultures. It is worthy of mention that phages have also been isolated from other food fermentations (e.g., sauerkraut, coffee, and wine), but their role in those situations is not as destructive as in the dairy environment due to its liquid nature, its relatively short fermentation time scale, and the large volumes of milk involved, usually with multiple successive

Figure 9.1 Transmission electron micrographs of two lytic bacteriophage particles (phage TP-J34; see arrows) adsorbed to cells of *Streptococcus thermophilus* culture, illustrating the beginning of phage infection cycle. Phage particle in micrograph (a) still contains DNA in the head, while that in micrograph (b) has already ejected the DNA into host cell. Note also the morphological change of surface of the infected cell in micrograph (b). Phage-induced cell lysis is expected to occur in approximately half an hour after phage adsorption (see Figure 9.2).

Figure 9.2 Scanning electron micrograph demonstrating the deleterious effect of bacteriophage infection in an *S. thermophilus* culture. Arrows indicate cells that have already lysed and released their cell content with new phage progeny. Phage particles are not visible at this low magnification but are shown in Figures 9.1 and 9.3.
uses of fermentation vats daily (Coffey and Ross 2002). Phage infections begin with adsorption of
the phage to receptor molecules on the bacterial cell surface (Figure 9.1), followed by injection and
replication of the phage genome, intracellular assembly of progeny phages, and finally cell lysis
with release of progeny phages (Figure 9.2). Research efforts in a variety of research laboratories
worldwide have focused on understanding the complex and dynamic mechanisms of phage–host
interactions as detailed below primarily using examples from L. lactis and Streptococcus thermophilus
where most of the phage resistance research in LAB has been performed.

9.2 Bacteriophages of LAB

Bacteriophages of LAB belong to the order Caudovirales. Phages in this particular order are char-
acterized by a capsid connected to a tail and double-stranded DNA genomes (Ackermann 1998).
The phages can be further divided into three families based on their tail morphology; Siphoviridae
(long noncontractile tail), Myoviridae (long contractile tail), and Podoviridae (short noncontrac-
tile tail) (Figure 9.3). The nature and size of the phage head is also an important element in

Figure 9.3 Transmission electron micrographs of dairy bacteriophages demonstrating various
morphotypes. Myoviridae phage (Lactobacillus gasseri phage Lgal) shown with extended (1a) and
contracted (1b) tail sheath (Ismail et al. 2009). Siphoviridae phages with noncontractable tails are
illustrated by L. lactis phages BK5-T (2), TP901-1 (3), P335 (4), r1t (5), P087 (6), P008 (7), P001 (9),
1358 (10) (Deveau et al. 2006), and by the Leuconostoc mesenteroides phage P770 (11) (Atamer
et al. 2011). A tail-less Podoviridae phage is shown in micrograph 8 (L. lactis phage P026). Phages
are characterized by their different tail lengths, tail appendices, and baseplate structures and are
genetically unrelated. Genomes are packed in either isometric (1a and 1b, 2–7, 10–11) or prolate
(8–9) phage heads. Phages have either temperate (top row) or virulent (bottom row) lifestyle.
Phage morphology (Figure 9.3). Phages belonging to morphotype 1 have an isometric head, those belonging to morphotype 2 have a small prolate head, and phages belonging to morphotype 3 have elongated or large prolate heads. Phages are thus further classified into species/groups on the basis of morphological similarities and homology across DNA sequences. Interestingly, lactococcal phages isolated to date belong to either Siphoviridae or Podoviridae families. Lactobacillus phages have been found for all three families, whereas all S. thermophilus phages isolated to date belong to the Siphoviridae family.

Genome sequencing alongside molecular techniques have therefore completely reinvigorated our understanding of LAB bacteriophages, resulting in valuable classification schemes and rapid molecular methods for phage detection, including multiplex PCR and quantitative real-time PCR assays (del Rio et al. 2007; Binetti et al. 2008; del Rio et al. 2008; Martin et al. 2008; Verreault et al. 2011). LAB bacteriophage genomes tend to be organized into distinct modular regions involved in morphogenesis, lysis, replication, transcription, and lysogeny. Bacteriophage genomes are constantly evolving through point mutations, deletions, and genetic exchange events (Brüssow et al. 1998). The following sections discuss phages of L. lactis and S. thermophilus, which have been completely sequenced in terms of their classification and evolution (for a review of Lactobacillus phages, see Villion and Moineau 2009).

### 9.2.1 Genomics of Lactococcus lactis Bacteriophages

Until recently lactococcal phages were classified into 12 groups based on similarities across morphological features and DNA homologies. In 2006 Deveau and co-workers reassessed the classification scheme so that lactococcal phages are now divided into 10 genetically distinct groups (Table 9.1), although only three of these are particularly problematic for milk fermentations globally, namely 936, c2, and P335 (Deveau et al. 2006). Phages of the latter group may be temperate or virulent whereas members of the 936 and c2 groups are virulent. While phages of the other seven groups have been rarely encountered in failed fermentations to date, investigations into their genome biology can also provide important insights into phage evolution in the dairy setting.

To date 12 lactococcal phage genomes belonging to the 936 group have been completely sequenced (Table 9.1). Interestingly, Rousseau and Moineau (2009) sequenced six of these genomes and proposed a core genome for the 936 group consisting of 33 open reading frames (ORFs) that mostly encode structural proteins. Indeed, a level of 62.2–99.5% identity at the nucleotide level was observed across all phage genomes of the 936 group. The six phages analyzed by Rousseau and Moineau were isolated over a 9-year period from a Canadian cheese factory and hence provide some insight into the evolution and persistence of 936-type phages in the dairy setting. The isolation of the same phage after a 14-month period demonstrates the ability of these phages to persist successfully in the dairy environment for a long time. Comparative genomic analysis suggested that the more recently isolated phages evolved from the older phage isolates. However, the unknown phage DNA in the more recent isolates was not derived from prophage DNA encoded in the host strains; hence, the authors suggest that some phage modules were swapped with other virulent phages in the plant. A recent study investigating the abundance of lactococcal phages in eight Norwegian dairies producing Dutch-type cheese demonstrated that members of the 936 group of phages were also the predominant group, even at the bulk starter stage (Kleppe et al. 2011). As phage levels in raw milk samples were low, the authors proposed that the phages came from within the plant, highlighting the evolved capacity of this group of phages to successfully survive in the dairy environment (Kleppe et al. 2011).
Table 9.1 Phages and Prophages of *L. lactis* with Completed Genome Sequence Analysis

<table>
<thead>
<tr>
<th>Phage</th>
<th>Life Cycle</th>
<th>Species</th>
<th>Genome Size (kb)</th>
<th>Reference</th>
</tr>
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<td>22.195</td>
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<tr>
<td>c2</td>
<td>Virulent</td>
<td>c2-like</td>
<td>22.172</td>
<td>Lubbers et al. (1995)</td>
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<tr>
<td>sk1</td>
<td>Virulent</td>
<td>936-like</td>
<td>28.451</td>
<td>Chandry et al. (1997)</td>
</tr>
<tr>
<td>bIL170</td>
<td>Virulent</td>
<td>936-like</td>
<td>31.754</td>
<td>Crutz-Le Coq et al. (2002)</td>
</tr>
<tr>
<td>bIBB29</td>
<td>Virulent</td>
<td>936-like</td>
<td>29.305</td>
<td>Hejnowicz et al. (2009)</td>
</tr>
<tr>
<td>SL4</td>
<td>Virulent</td>
<td>936-like</td>
<td>28.144</td>
<td>Rousseau and Moineau (2009)</td>
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<tr>
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<td>Virulent</td>
<td>936-like</td>
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<td>jj50</td>
<td>Virulent</td>
<td>936-like</td>
<td>27.453</td>
<td></td>
</tr>
<tr>
<td>P008</td>
<td>Virulent</td>
<td>936-like</td>
<td>28.538</td>
<td></td>
</tr>
<tr>
<td>BK5-T</td>
<td>Temperate</td>
<td>P335-like</td>
<td>40.003</td>
<td>Desiere et al. (2001)</td>
</tr>
<tr>
<td>ΦLC3</td>
<td>Temperate</td>
<td>P335-like</td>
<td>32.172</td>
<td>Blatny et al. (2004)</td>
</tr>
<tr>
<td>r1t</td>
<td>Temperate</td>
<td>P335-like</td>
<td>33.350</td>
<td>van Sinderen et al. (1996)</td>
</tr>
<tr>
<td>Tuc2009</td>
<td>Temperate</td>
<td>P335-like</td>
<td>38.347</td>
<td>Seegers et al. (2004)</td>
</tr>
<tr>
<td>TP901-1</td>
<td>Temperate</td>
<td>P335-like</td>
<td>37.667</td>
<td>Brondsted et al. (2001)</td>
</tr>
<tr>
<td>ul36</td>
<td>Virulent</td>
<td>P335-like</td>
<td>36.798</td>
<td>Labrie and Moineau (2002)</td>
</tr>
<tr>
<td>bIL285</td>
<td>Inducible phage</td>
<td>P335-like</td>
<td>35.538</td>
<td>Chopin et al. (2001)</td>
</tr>
<tr>
<td>bIL286</td>
<td>Inducible phage</td>
<td>P335-like</td>
<td>41.834</td>
<td></td>
</tr>
<tr>
<td>bIL309</td>
<td>Inducible phage</td>
<td>P335-like</td>
<td>36.949</td>
<td></td>
</tr>
<tr>
<td>bIL310</td>
<td>Inducible phage</td>
<td>n.r.</td>
<td>14.957</td>
<td></td>
</tr>
<tr>
<td>bIL311</td>
<td>Noninducible prophage</td>
<td>n.r.</td>
<td>14.510</td>
<td></td>
</tr>
<tr>
<td>bIL312</td>
<td>Inducible prophage</td>
<td>n.r.</td>
<td>15.179</td>
<td></td>
</tr>
<tr>
<td>P335</td>
<td>Virulent</td>
<td>P335-like</td>
<td>33.613</td>
<td>Labrie et al. (2008)</td>
</tr>
<tr>
<td>4268</td>
<td>Virulent</td>
<td>P335-like</td>
<td>36.596</td>
<td>Trotter et al. (2006)</td>
</tr>
</tbody>
</table>

(continued)
Unlike phages of the 936 group, members of the P335 phage group are much more diverse in terms of genetic relatedness, sharing only 10–33% homology at the sequence level (Chopin et al. 2001; Labrie and Moineau 2002) and have been described as a polythetic species (Deveau et al. 2006). This high degree of mosaicism is presumably related to the ability of this phage group to undergo extensive genetic recombination events between incoming phages and resident prophage sequences in host strains. Indeed, the P335 phage ul36 has demonstrated a marked capability to undergo genomic reshuffling. For example, homologous recombination between phage ul36 and a remnant prophage on the chromosome of the host strain *L. lactis* SMQ-88 resulted in two new recombinant phage mutants that were resistant to abortive infection mechanism AbiK and one exhibited a reduced burst size and new origin of replication (Bouchard and Moineau 2000). Phage ul37 evolved from a genetic exchange event between phage ul36 and its host *L. lactis* NCK203 (Moineau et al. 1994). The resulting phage had a longer tail, a different base plate, and a new origin of replication, and was resistant to the abortive infection mechanism of the host strain. The plasticity of the phage ul36 genome was again highlighted when four phage mutants (resistant to AbiK, AbiT, or both) evolved following infection of the host strain *L. lactis* SMQ-86 with the phage (Labrie and Moineau 2007). Genetic analysis revealed that the four new phages evolved following homologous and nonhomologous recombination events with a resident prophage in the host strain and as much as 79% of the genome was exchanged in one mutant phage. Deveau et al. (2006) suggest that such genome plasticity is a strategy for adapting to new hosts and evading new phage resistance mechanisms. Interestingly, the genome of phage P335, the type phage of the P335 species, was shown to contain four moron genes with homology to genes from *Enterococcus faecium* and *Streptococcus pyogenes*, which occurred downstream of the early expressed genes (Labrie et al. 2008). Transcriptional studies revealed that the four genes were autonomously expressed. The fact that three of the genes were homologous to *S. pyogenes* prophage sequences suggests that the region was acquired by phage P335 from another phage genome (Labrie et al. 2008).

A proteomic phylogenetic tree consisting of phages from the 10 known lactococcal phage groups perhaps unsurprisingly revealed the existence of three subgroups within the P335 phage species (subgroup I = P335, Tuc2009, TP901-1, ul36; subgroup II = BK5-T, 4268, bIL286;
subgroup III = r1t, φLC3) while the P335-like prophages bIL285, bIL309, and φSMQ-86 grouped separately from each other and the other P335 subgroups (Samson and Moineau 2010). However, Labrie et al. (2008) suggested that grouping the P335-like phages as the polythetic P335 species may be more practical given the extent of genome reshuffling displayed by this group of phages.

Only two complete genomes are currently available for the c2 group of lactococcal phages, phage c2 and phage bIL67 (Table 9.1), which share 80% sequence identity at the nucleotide level (Lubbers et al. 1995). Interestingly, phages of the c2 group were not detected in any of the eight Norwegian dairies producing Dutch-type cheese (Kleppen et al. 2011). The authors proposed that the absence of c2-type phages may be linked to their sensitivity to pasteurization (Madera et al. 2004) or that starter culture manufacturers may have selected for strains that harbor mutations in the pip gene encoding the receptor required for phage c2 infection (Geller et al. 1993; Babu et al. 1995; Garbutt et al. 1997). However, c2-type phages were isolated from whey samples from three Slovenian dairy plants over a 2-year period (Miklic and Rogelj 2003). Phages belonging to the c2 group were also found to be abundant in milk plants in various parts of Poland (Szczepanska et al. 2007) and in dairy products from various regions within the Republic of Belarus (Raiski and Belyasova 2009). Moreover, a recent study using real-time PCR to determine the presence of airborne lactococcal phages in a cheese manufacturing plant demonstrated that c2-type and 936-type phages were present in the air at concentrations of at least 10^3 genomes per cubic meter (Verreault et al. 2011). Surfaces were also found to contain c2-type and 936-type phages at concentrations of 10^3 genome copies per square centimeter.

The remaining sequenced lactococcal phage genomes belong to phages that have been rarely found in dairy plants to date and as such have been designated the representative phages of the remaining lactococcal phage species (Table 9.1). Phage Q54 was isolated from a failed sour cream production (Fortier et al. 2006). The ORFs encoded by the Q54 genome generally shared very low homology with proteins in databases, and hence Q54 represents a new phage species. However, 22–34% amino acid identity was observed between phage Q54 and c2-like phages, and a few early proteins displayed similarity to early expressed proteins from 936-like phages. The authors postulated that phage Q54 may have evolved from past recombination events involving 936- and c2-like phages. Moreover, three putative ORFs of Q54 showed a degree of relatedness to proteins from P335-like phages, suggesting recombination events with host-encoded prophage sequences. The phage also demonstrated an unusual +1 translational frameshifting in the tail structural genes.

Phage 1706 was isolated from a failed cheese production in France (Garneau et al. 2008). It displayed limited similarity with other sequenced phages, although 8 of its 76 ORFs were found to share homology with P335-like phages. The most surprising observation was the high level of homology observed between 21 predicted proteins of 1706 and ORFs of Ruminococcus torques and/or Clostridium leptum, both Firmicutes, which are members of the human gut microflora. The authors postulated that phage 1706 may have evolved from past recombination events involving 936- and c2-like phages. Moreover, three putative ORFs of Q54 showed a degree of relatedness to proteins from P335-like phages, suggesting recombination events with host-encoded prophage sequences. The phage also demonstrated an unusual +1 translational frameshifting in the tail structural genes.

Phage 1358 was isolated from a dairy environment in New Zealand (Jarvis 1984). The uniqueness of this phage was observed in its GC content, which was calculated at 51% and displayed a bias for GC-rich codons (Dupuis and Moineau 2010). The GC content of lactococcal phages has been shown to range from 33% (phage 1706) to 37% (phage KSY1). Moreover, 34.9% of the ORFs encoded on the 1358 genome displayed highest homology with ORFs encoded by the
Listeria phages P35 and P40, which were predicted to be involved in cell lysis or to be structural proteins. Interestingly, a study of six Listeria phages demonstrated that phages P35 and P40 also formed a distinct group clustering in a separate branch of a phylogenetic tree (Dorscht et al. 2009). The authors postulated that phage 1358 may be derived from a phage infecting Listeria that acquired genes to enable it to infect lactococcal hosts or alternatively the Listeria phages P35 and P40 evolved from a lactococcal phage (Dorscht et al. 2009). Considering that Listeria monocytogenes and L. lactis species can share some common environments, including milk and cheeses, the necessary contact for genetic exchange events between their phages is not an impossibility (Dupuis and Moineau 2010).

Phage 949 was isolated from cheese whey in New Zealand (Jarvis 1977). It has the lowest GC content (32.7%) calculated for any lactococcal phage to date and the largest genome (114,768 bp) and its 154 ORFs share limited homology with sequences in the database (Samson and Moineau 2010). Moreover, the genome contains six tRNAs and one group I intron.

Phage asccp28 is a member of the P034 phage species and was isolated from an Australian cheese factory (Kotsonis et al. 2008). It belongs to the Podoviridae family and more closely resembles φ29-like phages of Bacillus subtilis and phage Cp-1 of Streptococcus pneumoniae than lactococcal phages. Interestingly, although phages belonging to the P034 species are rarely encountered in the dairy industry, four phages belonging to the P034 species were recently isolated from dairy products in the Republic of Belarus from a total of 23 distinct lactococcal phages, suggesting that P034-like phages may be evolving a capacity to proliferate successfully in the domestic dairy environment (Raiski and Belyasova 2009).

The virulent phage KSY1 was originally isolated from spoiled “Viili,” a Finnish fermented ropy milk and also belongs to the Podoviridae family (Chopin et al. 2007). Interestingly, the phage has an unusually long capsid and sequences involved in the distal part of its tail were found to be more than 80% identical to gene segments from phages of the P335 group, suggesting that this region may have been acquired during genome reshuffling events with phages of the P335 group. The phage also contains a T7-like transcription unit, suggesting that the phage uses a transcription strategy similar to phage T7.

9.2.2 Genomics of Streptococcus thermophilus Bacteriophages

Unlike L. lactis phages S. thermophilus phages tend to be more homogenous, and it has been proposed that such phages are derived from a common ancestor (Mercenier 1990) and belong to one polythetic species consisting of both temperate and virulent phages (Brüssow and Desiere 2001; Deveau et al. 2008). They are currently divided into two distinct groups or subspecies based on their mode of packaging where cos-type phages contain two major structural proteins and pac-type phages have been shown to contain three (Le Marrec et al. 1997). To date 11 complete genome sequences are available for S. thermophilus phages (Table 9.2). Bacteriophage 5093 is the most diverse in terms of genome sequence and possibly represents a third type of S. thermophilus phage (Mills et al. 2011). Quiberoni et al. (2010) devised a core genome for 10 of the sequenced S. thermophilus phages (excluding phage 5093). Indeed, using phage DT1 the core genome of S. thermophilus phages consists of four ORFs and is defined as follows: an ORF of unknown function (ORF21), an endolysin (ORF25), an endolysin (ORF27), a putative DNA-binding protein (ORF42), and an ORF of unknown function (ORF44). Core genomes were also established for the cos- and pac-type groups individually consisting of 26–27 ORFs for cos-type phages and 25–26 ORFs for pac-type phages (Quiberoni et al. 2010).
9.3 Bacteriophage Resistance

While phage research in general has recently enjoyed somewhat of a renaissance, phage-related issues have been a central theme in LAB research over the last 30 years. Indeed, phage infection continues to plague even modern-day dairy plants, albeit complete vat failure as a result of phage infection is a rarity. However, phages are still responsible for inferior product formation, a situation generally associated with slow vats where acidity development is suboptimal (Coffey and Ross 2002). The understanding and correct exploitation of phage resistance mechanisms are vital for the development of robust LAB starter cultures that can withstand phage infection and consistently produce desirable products.

As a result of the coevolutionary arms race between bacteria and phages, bacteria have developed/acquired numerous mechanisms to evade phage attack, and the LAB are no exception. Many of these mechanisms have been identified on plasmids, particularly for lactococci (Mills et al. 2006), while others exist on the chromosome. Plasmid-encoded mechanisms can be particularly functional if the plasmid can be mobilized to other strains via food-grade methods. These phage resistance mechanisms can be categorized as phage adsorption inhibition, inhibition of phage DNA injection, restriction/modification (R/M), Abi, and the clustered regularly interspaced short palindromic repeat (CRISPR)–Cas system. Moreover, various artificial phage resistance mechanisms have also been designed that interfere with different stages of the phage life cycle (for a review of these mechanisms, see Sturino and Klaenhammer 2006).

9.3.1 Prevention of Phage Adsorption

At the initial stage of infection the phage must attach to a suitable receptor on the cell wall surface before the injection of phage DNA. These receptors have been linked to carbohydrates such as rhamnose, glucose, galactose, glucosamine, and ribose in certain lactococci, streptococci, and

<table>
<thead>
<tr>
<th>Phage</th>
<th>Life Cycle</th>
<th>Group</th>
<th>Genome Size (kb)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>O1205</td>
<td>Temperate</td>
<td>pac</td>
<td>43.075</td>
<td>Stanley et al. (1997)</td>
</tr>
<tr>
<td>Sfi11</td>
<td>Virulent</td>
<td>pac</td>
<td>39.807</td>
<td>Lucchini et al. (1999)</td>
</tr>
<tr>
<td>O58</td>
<td>Virulent</td>
<td>pac</td>
<td>35.543</td>
<td>Deveau et al. (2008)</td>
</tr>
<tr>
<td>2972</td>
<td>Virulent</td>
<td>pac</td>
<td>34.704</td>
<td>Levesque et al. (2005)</td>
</tr>
<tr>
<td>ALQ13.2</td>
<td>Virulent</td>
<td>pac</td>
<td>35.525</td>
<td>Guglielmotti et al. (2009)</td>
</tr>
<tr>
<td>DT1</td>
<td>Virulent</td>
<td>cos</td>
<td>34.815</td>
<td>Tremblay and Moineau (1999)</td>
</tr>
<tr>
<td>Sfi19</td>
<td>Virulent</td>
<td>cos</td>
<td>37.370</td>
<td>Desiere et al. (1998)</td>
</tr>
<tr>
<td>Sfi21</td>
<td>Temperate</td>
<td>cos</td>
<td>40.739</td>
<td>Desiere et al. (1998)</td>
</tr>
<tr>
<td>7201</td>
<td>Virulent</td>
<td>cos</td>
<td>35.466</td>
<td>Stanley et al. (2000)</td>
</tr>
<tr>
<td>Abc2</td>
<td>Virulent</td>
<td>cos</td>
<td>34.882</td>
<td>Guglielmotti et al. (2009)</td>
</tr>
<tr>
<td>5093</td>
<td>Virulent</td>
<td>cos</td>
<td>37.184</td>
<td>Mills et al. (2011)</td>
</tr>
</tbody>
</table>
lactobacilli (Ishibashi et al. 1982; Forde and Fitzgerald 1999; Quiberoni et al. 2000; Binetti et al. 2002). Adsorption inhibition (Ads) describes a phage resistance mechanism that prevents the attachment of the phage particle to the cell surface receptor and can often be induced through the generation of bacteriophage-insensitive mutants (BIMs) (Coffey et al. 1998). While the genetic process is not well understood, in Lactococcus this has been linked to nonspecific point mutations in chromosomal genes coding for cell receptors (Forde and Fitzgerald 1999). Interestingly a PCR-based genome scan of a phage-insensitive lactococcal derivative revealed mutations in genes associated with cell wall metabolism, transmembrane and membrane-associated proteins, prophage components, transcriptional regulators, and enzymes of basal metabolism when compared with the phage-sensitive parent strain (Schmidt et al. 2010). BIMs expressing the Ads phenotype can be generated by exposing the bacterial culture to high phage numbers, a choice method in many laboratories due to its simplicity (Mills et al. 2007), although its success tends to be strain dependent. A number of lactococcal plasmids have also been associated with the Ads phenotype such as pME0030 (Sanders and Klaenhammer 1983), pSK112 (de Vos et al. 1984), and pCI528 (Lucey et al. 1992). In many cases these plasmids have been connected to increased levels of galactose and/or rhamnose on the cell surface. A plasmid-encoded 30-kDa cell wall protein was also shown to prevent the attachment of phage particles to the cell surface in L. lactis (Akcelik and Tunail 1992). However, phages can evolve to recognize different cell receptors and evade the Ads mechanism through genome modification. For example, S. thermophilus host range phage mutants, with expanded host specificity, were shown to contain point mutations in three putative tail proteins, including the phage receptor binding protein (Duplessis et al. 2006).

### 9.3.2 Inhibition of Phage DNA Entry

Following successful attachment of the phage particle to the cell surface receptor, the phage DNA is injected into the cell cytoplasm. Superinfection exclusion (Sie) systems are membrane-associated proteins that prevent the entry of DNA into the host cell and are often found encoded on prophage sequences in the host genome. The first Sie system to be discovered was initially found on the genome of the temperate lactococcal phage Tuc2009, called Sie2009 (McGrath et al. 2002). Although Tuc2009 belongs to the P335 lactococcal phage group, as do most lactococcal prophages, it confers immunity to the genetically distinct 936 group of phages, the predominant group of L. lactis-specific phages found in the dairy industry. A Sie-like system was also identified within the lysogeny module of the S. thermophilus temperate phage TP-J34 (Sun et al. 2006). The protein responsible for injection blocking was linked to a signal-peptide-bearing 142 amino acid lipoprotein, termed LTP, which was also capable of conferring resistance to some lactococcal phages when transformed into Lactococcus.

There has only been one report to date of a plasmid-encoded injection blocking mechanism, which has been linked to the lactococcal plasmid pNP40 (Garvey et al. 1996). However, despite the availability of the complete nucleotide sequence of pNP40, the genetic determinants encoding injection blocking have not been identified (O’Driscoll et al. 2006). The plasmid already contains two Abi systems (AbiE and AbiF) and the temperature-sensitive R/M system (LlaJ1), which have been suggested to act synergistically, hence resulting in the enhanced resistance encoded on pNP40.

### 9.3.3 R/M Systems

R/M systems are a ubiquitous group of proteins that are carried by most if not all bacteria (Labrie et al. 2010) and several such systems have been identified in the LAB (Table 9.3). These systems
<table>
<thead>
<tr>
<th>Name</th>
<th>Type</th>
<th>Host</th>
<th>Specificity(^a)</th>
<th>Location</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lla1403I</td>
<td>I</td>
<td>LL IL1403</td>
<td>Undetermined</td>
<td>Chromosome</td>
<td>Schouler et al. (1998b)</td>
</tr>
<tr>
<td>Lla42ol(^b)</td>
<td>I</td>
<td>LC IL420</td>
<td>Undetermined</td>
<td>pIL2614</td>
<td>Schouler et al. (1998a)</td>
</tr>
<tr>
<td>Lla82I</td>
<td>I</td>
<td>LL DPC220</td>
<td>Undetermined</td>
<td>pAH82</td>
<td>O’Sullivan et al. (2000)</td>
</tr>
<tr>
<td>Lla90I</td>
<td>I</td>
<td>LL DPC721</td>
<td>Undetermined</td>
<td>pAH90</td>
<td>O’Sullivan et al. (2000)</td>
</tr>
<tr>
<td>Lla90II</td>
<td>I</td>
<td>LL DPC721</td>
<td>Undetermined</td>
<td>pAH90</td>
<td>O’Sullivan et al. (2000)</td>
</tr>
<tr>
<td>LldI</td>
<td>I</td>
<td>LL LD10-1</td>
<td>Undetermined</td>
<td>pND861</td>
<td>Deng et al. (2000)</td>
</tr>
<tr>
<td>LlaGI</td>
<td>I SP</td>
<td>LC W10</td>
<td>5’ CTNGAYG 3’</td>
<td>pEW104</td>
<td>Madsen and Josephsen (2001); Smith et al. (2009)</td>
</tr>
<tr>
<td>LlaBIII</td>
<td>I (var)</td>
<td>LC W56</td>
<td>Undetermined</td>
<td>pJW566</td>
<td>Josephsen and Vogensen (1989); Kong et al. (2002)</td>
</tr>
<tr>
<td>ScrFI</td>
<td>II</td>
<td>LC UC503</td>
<td>5’ CC(\downarrow)NGG 3’</td>
<td>Chromosome</td>
<td>Fitzgerald et al. (1982); Davis et al. (1993); Fitzgerald et al. (1995); Twomey et al. (1997)</td>
</tr>
<tr>
<td>LlaI</td>
<td>II</td>
<td>LL ME2</td>
<td>Undetermined</td>
<td>pTR2030</td>
<td>Hill et al. (1989); O’Sullivan et al. (1995); O’Sullivan and Klaenhammer (1998)</td>
</tr>
<tr>
<td>Lla497I</td>
<td>II</td>
<td>LL NCDO497</td>
<td>5’ CCW(\downarrow)GG 3’</td>
<td>unspecified</td>
<td>Mayo et al. (1991)</td>
</tr>
<tr>
<td>LlaAI</td>
<td>II</td>
<td>LC W9</td>
<td>5’ (\downarrow)GATC 3’</td>
<td>pFW094</td>
<td>Nyengaard et al. (1993), (1995); Gabs and Josephsen (2003)</td>
</tr>
<tr>
<td>LlaDCHI</td>
<td>II</td>
<td>LC DCH-4</td>
<td>5’ GATC 3’</td>
<td>pSRQ700</td>
<td>Moineau et al. (1995); Boucher et al. (2001)</td>
</tr>
<tr>
<td>LlaCI</td>
<td>II</td>
<td>LC W15</td>
<td>5’ A(\downarrow)AGCTT 3’</td>
<td>pAW153</td>
<td>Josephsen et al. (1998); Madsen and Josephsen (1998a); Mruk et al. (2003)</td>
</tr>
</tbody>
</table>

(continued)
Table 9.3 R/M Systems Characterized from *L. lactis* and *S. thermophilus* (Continued)

<table>
<thead>
<tr>
<th>Name</th>
<th>Type</th>
<th>Host</th>
<th>Specificity&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Location</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
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<td>LlaDII</td>
<td>II</td>
<td>LC W39</td>
<td>5’ GC↓NGC 3’</td>
<td>pHW393</td>
<td>Madsen and Josephsen (1998b); Christensen and Josephsen (2004)</td>
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<tr>
<td>LlaFl</td>
<td>III</td>
<td>LL 42-1</td>
<td>Undetermined</td>
<td>pND801</td>
<td>Su et al. (1999)</td>
</tr>
<tr>
<td>LlaJII</td>
<td>II-Subtype</td>
<td>LL DRC3</td>
<td>5’ GAGGC 3’</td>
<td>pNP40</td>
<td>O’Driscoll et al. (2004, 2006)</td>
</tr>
<tr>
<td>SthSFil</td>
<td>I</td>
<td>ST Sfi1</td>
<td>Undetermined</td>
<td>Chromosome</td>
<td>Lucchini et al. (2000)</td>
</tr>
<tr>
<td>Sth135I</td>
<td>I</td>
<td>ST 135</td>
<td>Undetermined</td>
<td>pER35</td>
<td>Solow and Somkuti (2001)</td>
</tr>
<tr>
<td>Sth134I</td>
<td>II</td>
<td>ST 134</td>
<td>5’ C↓CGG 3’</td>
<td>Chromosome</td>
<td>Solaiman and Somkuti (1990)</td>
</tr>
<tr>
<td>Sth117I</td>
<td>II</td>
<td>ST 117</td>
<td>5’ CC↓WGG 3’</td>
<td>Chromosome</td>
<td>Solaiman and Somkuti (1991)</td>
</tr>
<tr>
<td>Sssl</td>
<td>II</td>
<td>ST T</td>
<td>5’ CCWGG 3’</td>
<td>Chromosome</td>
<td>Benbadis et al. (1991)</td>
</tr>
<tr>
<td>Sth455I</td>
<td>II</td>
<td>ST CNRZ455</td>
<td>5’ CCWGG 3’</td>
<td>Chromosome</td>
<td>Guimont et al. (1993)</td>
</tr>
<tr>
<td>Sth132I</td>
<td>II</td>
<td>ST 132</td>
<td>5’ CCCG(N)4 3’ 3’ GGGC(N)8 5’</td>
<td>Chromosome</td>
<td>Poch et al. (1997)</td>
</tr>
<tr>
<td>Sth368I</td>
<td>II</td>
<td>ST CNRZ368</td>
<td>5’ GATC 3’</td>
<td>Chromosome</td>
<td>Burrus et al. (2001); Bellanger et al. (2009)</td>
</tr>
<tr>
<td>Sth0I</td>
<td>II</td>
<td>ST 0</td>
<td>Undetermined</td>
<td>pSt0</td>
<td>Geis et al. (2003); Accession no. AJ242480</td>
</tr>
<tr>
<td>Sth8I</td>
<td>II</td>
<td>ST 8</td>
<td>Undetermined</td>
<td>pSt08</td>
<td>Geis et al. (2003); Accession no. AJ239049</td>
</tr>
</tbody>
</table>


Note: LC, *L. lactis* subsp. cremoris; LL, *L. lactis* subsp. lactis; ST, *Streptococcus thermophilus*; SP, single polypeptide; W = A or T; R = A or G; Y = C or T; N = ACG or T.

<sup>a</sup> The cleavage point where known is indicated by ↓.

<sup>b</sup> Designated Lla2614I in REBASE.NEB.COM.
function by digesting unmethylated foreign DNA with a dedicated restriction enzyme, while the methylated host DNA remains intact, although to a lesser extent, the foreign DNA may be methylated by the bacterial methylase. The fate of foreign DNA is determined by the processing rates of these two enzymes, but as the restriction enzyme is often more active than the methylase, the incoming foreign DNA is usually cleaved, while the host DNA is always methylated by the methylase (Labrie et al. 2010). The R/M systems of LAB have been classified into three main groups (type I–type III), which can also contain subclasses, based on molecular structure, sequence recognition, cleavage position, and the cofactors required (Bickle and Krüger 1993; Pingoud 2004). For example, type I R/M systems consist of three subunits: HsdR, restriction endonuclease (digests foreign DNA); HsdM, methylase (protects the host DNA); HsdS (specificity subunit of the endonuclease or the methylase). Interestingly, while many lactococcal plasmids encode R/M systems, several plasmids have also been shown to harbor an hsdS locus without the cognate hsdR and hsdM subunits. However, HsdS subunits by themselves can function in trans and are thus capable of interaction with HsdR and HsdM subunits encoded on other DNA elements (Schouler et al. 1998b; O’Sullivan et al. 1999, 2000; Boucher et al. 2001). Moreover, a resident hsdS gene on a 6-kb plasmid termed pAH33 was able to alter the specificity of a functional R/M system encoded on the 20.3-kb pAH82 plasmid, leading to increased phage resistance (O’Sullivan et al. 2000). This was facilitated by the integration of pAH33 into pAH82 via homologous recombination across the conserved sequences of the hsdS genes, resulting in a 26.5-kb cointegrate plasmid termed pAH90. Thus through HsdS shuffling, the efficiency of type I R/M systems can be vastly expanded to recognize different restriction sites. Most streptococcal R/M systems identified to date are located on the chromosome. However, the streptococcal type II R/M system, Sth368l, which is encoded on an integrative conjugative transposon called ICESt1, was successfully transferred by conjugation to other S. thermophilus strains as well as other bacterial species, including E. faecalis, S. pyogenes, and L. lactis subsp. cremoris (Burrus et al. 2001; Bellanger et al. 2009).

In typical predator–prey coevolutionary style, phages can avoid R/M through methylation of the phage genome, the accumulation of pinpoint mutations or deletions from the phage genome, or the uptake of DNA from the host genome. The latter phenomenon was first described when lactococcal phage 50 became completely resistant to the type II R/M system encoded on the lactococcal plasmid pTR2030. Indeed, the phage was able to boycott the R/M system after it acquired a copy of the plasmid-encoded methylase gene through an in vivo genetic exchange event (Hill et al. 1991). Moreover, the presence of R/M systems in Lactobacillus delbrueckii was shown to generate a modified/methylated phage progeny (derived from a prophage), which continued the infectious process (Suarez et al. 2009). The authors suggest that the presence of native phage resistance mechanisms and the occurrence of prophages in commercial host strains contribute strongly to diversify the phage population in a factory environment.

### 9.3.4 Abi Mechanisms

Abi mechanisms include a broad range of defenses that can interfere with genome replication, transcription, translation, packaging or assembly of phage particles, and come into play after the injection of phage DNA. They can interfere with one or two or all phage groups (Coffey and Ross 2002; Chopin et al. 2005). Death of the infected cell is the end result with the release of few or no phages (Chopin et al. 2005). Twenty-three Abi systems have been identified to date in Lactococcus ranging from AbiA to AbiZ (Table 9.4) and most are plasmid encoded. A single gene is generally responsible for the phenotype, although the involvement of two genes has been proposed (AbiE, G, L, and T). Interestingly, although AbiV is a chromosomally encoded mechanism, it was
### Table 9.4 Abortive Infection Systems Characterized from *L. lactis*

<table>
<thead>
<tr>
<th>Name</th>
<th>Source</th>
<th>Sequence Accession Number</th>
<th>Accession Number</th>
<th>Groups of Sensitive Phages</th>
<th>Effects on Phage Life Cycle</th>
</tr>
</thead>
<tbody>
<tr>
<td>AbiA</td>
<td>pTR2030</td>
<td>U17233</td>
<td>936, c2, P335</td>
<td></td>
<td>No DNA replication of P335 phages, interference with a phage recombinase</td>
</tr>
<tr>
<td></td>
<td>pCI823</td>
<td>AAA25159</td>
<td></td>
<td></td>
<td>Unknown</td>
</tr>
<tr>
<td>AbiB</td>
<td>–</td>
<td>M77708</td>
<td>936</td>
<td></td>
<td>Decay of phage transcripts</td>
</tr>
<tr>
<td></td>
<td>pH003</td>
<td>AF247159</td>
<td></td>
<td></td>
<td>Unknown</td>
</tr>
<tr>
<td>AbiC</td>
<td>pTN20</td>
<td>M95956</td>
<td>936, P335</td>
<td></td>
<td>Limits production of major capsid protein</td>
</tr>
<tr>
<td>AbiD</td>
<td>pBF61</td>
<td>U10992</td>
<td>936, c2</td>
<td></td>
<td>Unknown</td>
</tr>
<tr>
<td>AbiD1</td>
<td>pIL105</td>
<td>L35176</td>
<td>936, c2</td>
<td></td>
<td>Induction by a phage protein, interference with a phage RuvC-like endonuclease</td>
</tr>
<tr>
<td>AbiE</td>
<td>pNP40</td>
<td>U36837</td>
<td>936</td>
<td></td>
<td>Affects packaging of phage DNA</td>
</tr>
<tr>
<td>AbiF</td>
<td>pNP40</td>
<td>U36837</td>
<td>936, c2</td>
<td></td>
<td>Delays DNA replication of 936 phages</td>
</tr>
<tr>
<td>AbiG</td>
<td>pCI750</td>
<td>U60336</td>
<td>936, (c2), P335</td>
<td></td>
<td>Affects RNA transcription</td>
</tr>
<tr>
<td>AbiH</td>
<td>Chr</td>
<td>X97651</td>
<td>936, (c2)</td>
<td></td>
<td>Unknown</td>
</tr>
<tr>
<td>Abil</td>
<td>pND852</td>
<td>U38973</td>
<td>936, (c2)</td>
<td></td>
<td>Affects DNA packaging</td>
</tr>
<tr>
<td>Abil</td>
<td>pND859</td>
<td>U41294</td>
<td>(936)</td>
<td></td>
<td>Unknown</td>
</tr>
<tr>
<td>AbiK</td>
<td>pSRQ800</td>
<td>U35629</td>
<td>936, (c2), P335</td>
<td></td>
<td>No DNA replication of P335 phages, interference with a phage Erf or Rad52-like recombinase. Normal DNA replication of 936 phages</td>
</tr>
<tr>
<td>AbiL</td>
<td>pND861</td>
<td>U94520</td>
<td>936, (c2)</td>
<td></td>
<td>Unknown but may act at point of transcription</td>
</tr>
<tr>
<td>AbiN</td>
<td>prophage</td>
<td>Y11901</td>
<td>936, c2</td>
<td></td>
<td>Unknown</td>
</tr>
<tr>
<td>AbiO</td>
<td>pPF144</td>
<td>I61427</td>
<td>936, c2</td>
<td></td>
<td>Unknown</td>
</tr>
<tr>
<td>AbiP</td>
<td>pIL2614</td>
<td>U90222</td>
<td>936</td>
<td></td>
<td>Early arrest of DNA replication, absence of early transcripts switch-off</td>
</tr>
<tr>
<td>AbiQ</td>
<td>pSRQ900</td>
<td>AF001314</td>
<td>936, c2</td>
<td></td>
<td>Affects DNA packaging</td>
</tr>
<tr>
<td>AbiR</td>
<td>pKR223</td>
<td>AF216814</td>
<td>c2</td>
<td></td>
<td>Lowers DNA replication of c2 phages</td>
</tr>
<tr>
<td>AbiS</td>
<td>pAW601</td>
<td>AJ132009</td>
<td>(936)</td>
<td></td>
<td>Unknown</td>
</tr>
</tbody>
</table>

*(continued)*
Bacteriophage and Anti-Phage Mechanisms in Lactic Acid Bacteria

recently transferred successfully to another *L. lactis* strain, presumably via conjugation (Haaber et al. 2009).

Phages have also evolved clever strategies to evade Abi mechanisms (Coffey and Ross 2002). Most recently Labrie and Moineau (2007) demonstrated that prophage sequences and Abi mechanisms significantly influenced the genetic makeup of emerging lytic phages as previously discussed.

### 9.3.5 CRISPR–Cas Systems

CRISPRs have recently been shown to play a role in phage resistance, particularly for *S. thermophilus*. CRISPR loci consist of highly conserved repeats of approximately 21–48 bp, which are separated by variable sequences of constant and similar length, called spacers of approximately 20–58 bp (Grissa et al. 2007; Horvath et al. 2008; Deveau et al. 2010). The phage resistance of many *S. thermophilus* BIMs has been linked to the integration of novel spacers (short sequences from phage DNA) into the CRISPR loci in response to phage attack (Barrangou et al. 2007; Horvath et al. 2008; Mills et al. 2010). It has been proposed that these spacers function as small interfering RNAs, base pairing with target DNA or mRNAs; promoting their degradation or translation shutdown (Brouns et al. 2008; Marraffini and Sontheimer 2008; Sorek et al. 2008), thus resulting in termination of the phage lytic cycle. Multiple CRISPR families have been identified within the genomes of *Bifidobacterium*, *Lactobacillus*, and *Streptococcus*, and similar CRISPR loci were found in distant organisms, suggesting that these loci have been subjected to horizontal gene transfer and further evolved independently in select lineages, partly owing to the selective pressure of phage attack (Horvath et al. 2009). However, virulent phages are rapidly evolving through single nucleotide mutations as well as deletions, in response to CRISPR as recently shown by Deveau et al. (2008).

### Table 9.4 Abortive Infection Systems Characterized from *L. lactis* (Continued)

<table>
<thead>
<tr>
<th>Name</th>
<th>Source</th>
<th>Sequence Accession Number</th>
<th>Groups of Sensitive Phages$^a$</th>
<th>Effects on Phage Life Cycle</th>
</tr>
</thead>
<tbody>
<tr>
<td>AbiT</td>
<td>pED1</td>
<td>AF483000</td>
<td>936, P335</td>
<td>Lowers DNA replication of 936 and P335 phages</td>
</tr>
<tr>
<td>AbiU</td>
<td>pND001</td>
<td>AF88839</td>
<td>(936), c2, P335</td>
<td>Delays transcription of 936 and c2 phages</td>
</tr>
<tr>
<td>AbiV</td>
<td>Chr</td>
<td>AF324839</td>
<td>936, c2</td>
<td>Prevents cleavage of the replicated phage DNA of 936-like phages</td>
</tr>
<tr>
<td>AbiZ</td>
<td>pTR2030</td>
<td>U17233</td>
<td>936, P335</td>
<td>May interact cooperatively with holin to cause premature lysis</td>
</tr>
</tbody>
</table>


*Note:* Chr, chromosome.

$^a$ Groups in parentheses are relatively less sensitive.
9.4 Conclusion

Even in the 21st century, phage infection continues to challenge scientists and food manufacturers alike. However, the recent upsurge in genome sequencing alongside our in-depth knowledge of phage resistance mechanisms suggests that we have reached a new understanding of the molecular intricacies involved in phage–host interactions. One of the striking realizations from this is the rapid ability of phages to evolve in response to resistance mechanisms, which is often achieved by exploiting the mechanism itself or other host-encoded sequences. Thus the exploitation of single mechanisms should ideally be avoided but rather each cell should be equipped with a selection of phage resistance mechanisms targeting different stages of the phage infection process. This has previously been achieved in the laboratory whereby plasmids encoding Ads, R/M, and Abi were successfully stacked in one strain via food-grade gene transfer, which was then used as a single-strain starter for cheddar cheese manufacture (O’Sullivan et al. 1998). Such a hurdle-type approach should dramatically reduce the expanding phage diversity within dairy plants. Thus the search for novel mobile phage resistance mechanisms needs to continue to enable the construction of naturally phage-resistant strains for industrial-scale applications.

References


Chapter 10

Lactic Acid Bacteria in Vegetable Fermentations

Kun-Young Park and Boh Kyung Kim

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10.1 Introduction

Fermenting vegetables with salt is a widespread traditional practice for preserving and for improving flavor, texture, and health functionalities, usually without adding preserving agents. Sauerkraut, cucumber pickles, and olives are the most common fermented vegetables in the Western world. Kimchi is a Korean fermented vegetable dish that is popular in Korea and Japan, and is gaining acceptance in many Asian and Western countries.

Vegetables, especially cruciferous vegetables, are reported to exhibit health benefits. Various nutraceuticals found in vegetables and fermented end products can improve human health. Vegetables contain vitamin C, carotenoids, chlorophyll, flavonoids, dietary fiber, and are rich in minerals, including K, Mg, Se, which show protective effects against various degenerative diseases such as cancer, obesity, diabetes, and hypertension. Lactic acid bacteria (LAB)-fermented vegetables might exhibit better functionalities than the raw vegetables themselves. Probiotic LAB-fermented vegetable juices are also candidates for healthy beverages.

There are numerous studies on processing methods, fermentation, and microbiology of sauerkraut, cucumber, and olive. However, limited research has been done to support the putative health benefits of these products. The microbiology and processing methods of kimchi have also been studied, and the health functionalities of kimchi LAB and kimchi have recently been reported (Park et al. 1995; Cheigh and Park 1994; Park 1995; Park and Cheigh 2004; Park and Rhee 2005).

LAB have increased in popularity due to their role as probiotics that improve colon health. LAB may prevent colon cancer by alteration of the metabolic activities of intestinal microflora, binding and degrading potential carcinogens, production of antimutagenic and antitumorigenic compounds, enhancing the host’s immune response, and other actions (Rafter 2002). LAB-fermented vegetables have greater demonstrated functionality than the same raw vegetables (Park and Rhee 2005). Increased levels of LAB and fermentation end products combined with preexisting phytochemicals offer health benefits, and improve flavor and shelf life. Vegetables are usually fermented naturally by bacteria present in the environment; however, isolated LAB can be used as starter cultures for the development of functional vegetable products with greater consistency and improved functionality.
10.2 Vegetable LAB Strains and Microbial Changes during Vegetable Fermentation

There are two kinds of vegetable LAB. One is the homofermentative LAB that produce mainly lactic acid after fermentation. The other is heterofermentative LAB that produce lactic acid, CO₂, and acetate/ethanol at a 1:1:1 ratio (Ray and Bhunia 2008). Homolactic bacteria ferment glucose (hexose) through the Embden–Meyerhoff–Parnas (EMP) pathway. They have fructose diphosphate aldolase, which can hydrolyze a six-carbon glucose into two molecules of three-carbon fermented lactic acid. They do not carry phosphoketolase that is in heterofermentative LAB. Heterolactic bacteria lack fructose diphosphoaldolase for the EMP pathway but have glucose phosphate dehydrogenase and xylulose phosphoketolase, which metabolize glucose through the hexose monophosphate shunt (HMS) pathway (Ray and Bhunia 2008).

The major LAB found in fermented vegetables are as follows: *Lactobacillus acidophilus*, *Lb. lactis*, *Lb. leichmanii*, *Lb. salivarius*, *Lb. plantarum*, *Lb. sake*, *Streptococcus thermophilus*, *Pediococcus acidilactici*, *P. damnosus*, *P. pentosaceus*, *Enterococcus faecalis*, which are classified as homofermentative LAB, and *Leuconostoc mesenteroides*, *Ln. paramesenteroides*, *Ln. dextranicum*, *Lb. brevis*, *Lb. cellobiosus*, *Lb. confuses*, *Lb. fermentum*, which are classified as heterofermentative LAB (Ray and Bhunia 2008; Nout and Rombouts 1992).

*Lb. plantarum* and *Lb. sake* were classified as facultative heterofermentative LAB by Nout and Rombouts (1992). They believed that *Lb. plantarum* ferments glucose homofermentatively, but other carbohydrates can be fermented in a heterofermentative manner as well. However, these two LAB are usually classified as homofermentative LAB (Ray and Bhunia 2008). It is generally accepted that *Lb. plantarum* is a homofermentative LAB and produces higher amounts of lactic acid. *Leuconostoc* sp. and *Lb. fermentum* can also ferment pentose (fructose) sugars though the pentose phosphate (PP) pathway, and produce ATP, lactate, acetate, and ethanol. However, CO₂ is not produced by the PP pathway.

Vegetables normally contain gram-negative aerobic bacteria, yeasts, and gram-positive LAB. When salt is added under anaerobic conditions, LAB can overgrow the aerobic bacteria and yeasts. The growth of LAB depends on the changes in the chemical and physical environments, such as variations in raw materials, available nutrients, salt concentration, O₂ concentration, temperature, pH, and other factors. Gram-negative bacteria, yeasts, and molds are suppressed during early-stage fermentation by the addition of salt or during the brining. Choi et al. (1991) reported that the numbers of total aerobic bacteria, and yeasts and molds were decreased by 11–16- and 29–87-fold, respectively; however, LAB counts increased by four times after brining cabbage (10 h in 10% brine).

Spoilage and pathogenic microorganisms can be removed during vegetable fermentation by the production of lactic or other organic acids, and by decreasing the pH (Cheigh and Park 1994). This has been demonstrated through studies of the sequential growth of LAB during sauerkraut fermentation (Harris 1998). Heterofermentative LAB, especially *Ln. mesenteroides*, can initially grow at higher pH. The pH usually starts at about 6.3 in the first stage of the fermentation. When pH is decreased to about 4.5, the growth of *Leuconostoc* species becomes inhibited and more acid-tolerant LAB, homofermentative LAB (e.g., *Lb. plantarum*), predominate and produce lactic acid, which decreases the pH to 4.0 or less (Harris 1998). Thus the major LAB in sauerkraut fermentation are *Ln. mesenteroides* and *Lb. plantarum*.

Kimchi is usually fermented at low temperature (5°C). Kimchi fermentation is dominated by *Leuconostoc* sp. at 5°C, but *Lactobacillus* sp. are the major LAB when fermentation occurs at 25°C, or higher temperatures (Lim et al. 1989). As shown in Figure 10.1, the counts of *Pediococcus* sp. and
Streptococcus sp. were lower. Although Lactobacillus sp. and Leuconostoc sp. were the major LAB participating in the fermentation, the level of Leuconostoc sp. was much higher at 5°C than at 20°C fermentation (Lee et al. 1992). Cho et al. (2006) reported that they isolated 970 bacteria during kimchi fermentation in a kimchi refrigerator. The representative genera of 15 species were Lactobacillus, Leuconostoc, and Weissella. Ln. citrus and Ln. gasicomitatum predominated during the first growth stage; however, Weissella koreensis predominated during the second stage. The population dynamics appeared to be markedly influenced by the temperature and seasonal variations in raw materials.

10.3 Processing Methods and Microorganisms in Fermented Vegetables

10.3.1 Sauerkraut

10.3.1.1 Processing

In German, the word sauerkraut means acid cabbage. The acid is produced by the natural or starter lactic acid fermentation bacteria in the salted-shredded cabbages. Mild-flavored, sweet, and white cabbage is used as the main cabbage. The cabbage is thinly sliced 0.08–0.16 cm (long and finely cut shreds), and salt (2–2.5%) is sprinkled on the shredded cabbage. The salted cabbage is packed into
Lactic Acid Bacteria in Vegetable Fermentations

10.3.1.2 Microorganisms in Sauerkraut Fermentation

Weissella and Ln. citreum were found in the heterolactic phase of the fermentation, Lb. plantarum predominated in the late stage, and two LAB species expected to be present (P. pentosaceus and L. brevis) were apparently minor constituents of the microbiota of commercial sauerkraut fermentations (Harris 1998). The species of LAB found in commercial sauerkraut fermentations by DNA fingerprinting include Ln. citreum, Ln. argentinum, Lb. paraplantarum, Lb. coryniformis, Weissella sp., and a newly identified species, Ln. fallax (Johanningsmeier et al. 2007). Ln. mesenteroides when grown in cabbage juice had a shorter lag phase and a more rapid generation time than any of the other organisms associated with sauerkraut fermentation (Pederson 1979). Single or mixed starter cultures of Ln. mesenteroides, Lb. brevis, and Lb. plantarum improved the sauerkraut quality over noninoculated natural fermentation. Cabbage fermented with Ln. mesenteroides consistently resulted in sauerkraut with a firm texture and reduced off-flavors in 0.5–2.0% NaCl (Johanningsmeier et al. 2007). In one fermentation, a mineral salt (3.5 kg) containing 57% NaCl and 28% KCl, 12% Mg sulfate, 2% lysine HCl, and 1% silicon dioxide was prepared. The use of mineral salt with a low level of salt (0.5%) for cabbage (400 kg) fermentation with commercial starter of Ln. mesenteroides resulted in mild-tasting sauerkraut juice with good sensory and microbiological quality (Wiander and Ryhanen 2005). When Lb. curvatus 2775 and Lb. plantarum DSM20174 were used as starters, the production of biogenic amines was lowered during sauerkraut fermentation (Halasz et al. 1999).

10.3.2 Fermented Cucumber

10.3.2.1 Processing

Pickling cucumbers (Cucumis sativus) are harvested while immature. Fully ripened cucumbers are not suitable since they become too large, change their color, have mature seeds, and are too soft for commercial uses. Fleming et al. (1995a) reported that cucumber pickles are preserved by fermentation (40% of U.S. production), pasteurization (40%), and refrigeration (20%).

10.3.2.1.1 Fermented Pickles

Fermented pickles are called salt stock, genuine dill pickles, or fermented dill pickles. They are the product of bacterial fermentation and are flavored with dill, other spices, and salt brine. The pickles are covered with salt brine and undergo lactic acid fermentation. Cucumbers are put into brine with an initial salt concentration of 5–8% at an ambient temperature. Brine is often acidified with acetic acid to pH 4.5 to remove CO₂ and stimulate LAB growth. Potassium sorbate (0.035%) or acetic acid (0.16%) can be added to the brine to prevent growth of fungi that may cause softening of the cucumbers. Fermentation occurs at a salt concentration of 5%, at 20–27°C for 2–3 weeks. The pH becomes 3.3–3.5, and acidity 1.1%, after the fermentation. Fermented pickles are then desalted and processed into various products, including sweet and sour pickles, mixed pickles,
processed dills, or sliced pickles. The pickles can be pasteurized at 74°C for 15 min to increase shelf life (Harris 1998).

10.3.2.1.2 Pasteurized Pickles

Also called fresh pack, the cucumbers are acidified with vinegar to almost pH 3.7, spices are added, and the pickles are pasteurized at 74°C for 15 min. The salt concentration is 0–3% and sugar concentration is different depending on pickle type.

10.3.2.1.3 Refrigerated Dills

Refrigerated dill pickles are characterized by their fresh cucumber flavor and crunchy texture. No microbial growth is desirable, and it can be prevented by addition of vinegar, chemical preservative (sodium benzoate), and constant refrigeration at <5°C. Otherwise, pickles can be added to 5% NaCl brine along with dill and garlic, and the brined cucumbers are stored and fermented at 3–7°C for 3–6 months at low temperature and salt concentration, and acidity is 0.3–0.6% (Fleming et al. 1995b).

10.3.2.2 Microorganisms in the Fermentation

The major groups of LAB identified by PCR in salt-fermented cucumber were *Lactobacillus*, *Leuconostoc*, and *Pediococcus* (Etchells et al. 1964). *Ln. mesenteroides* plays a more important role at low temperatures and low salt concentrations and is the initial major bacteria. Then *P. cerevisiae*, *Lb. brevis*, and *Lb. plantarum* begin to dominate the fermentation. The fermentation is completed by *Lb. brevis* and *Lb. plantarum*. *Ln. mesenteroides*, *P. cerevisiae*, and *Lb. plantarum* are recognized as especially important in cucumber fermentation (Vaughn 1982).

In one study, *E. faecalis*, *Ln. mesenteroides*, *Lb. brevis*, and *P. pentosaceus* were inoculated to cucumber fermentation as starters. *Lb. plantarum* predominated in the late stage of cucumber fermentation regardless of the species of LAB used for inoculation, due to the greater acid tolerance of this strain (Pederson and Albury 1961). Cucumber fermentation without NaCl was successful in a laboratory experiment in which the cucumbers were blanched at 77°C for 3 min and brined in a calcium acetate buffer with *Lb. plantarum* (Fleming et al. 1995b).

10.3.3 Fermented Olive

10.3.3.1 Processing

It is known that Spain, Turkey, Italy, Morocco, and Greece are the top 5 olive-producing countries. The phenolic glucoside oleuropein is the major bitter component of the fruits. Oleuropein is removed by soaking the olives in water or brine, or through hydrolysis in a 1–2% NaOH solution during processing. The green color (chlorophyll) of olives changes to black (anthocyanins) during ripening. There are three kinds of olive processing methods reported (Harris 1998).

10.3.3.1.1 Lye-Treated Black Olives in Brine (California-Style Black Ripe Olives)

This method of producing canned black ripe olives was developed in California, USA. Most California olives are processed with this method. Green to cherry red olives are harvested. Initial salt brining concentration is 5.0–7.5%. Salt is added at intervals of one to several days to increase
the NaCl concentration to 7.5–9.0%. The lactic acid concentration is 0.4–0.45% after 4–6 weeks. Instead of brine, an acidulant solution containing 0.7% lactic acid, 1.0% acetic acid, 0.3% sodium benzoate, and 0.3% potassium sorbate can be used. The fresh or stored olives are first treated with three to five applications of 0.5–2.0% NaOH (lye) and the olives are aerated during lye treatment; the natural phenolic compounds are oxidized and polymerized, forming a black pigment. Lye is removed by changing the water twice daily for 3–4 days. Washing water is replaced by dilute brine (0.8–2.5% NaCl) for a further 2–4 days. The olives are then sorted, canned, and thermally processed.

10.3.3.1.2 Lye-Treated Green Olives (Spanish-Style Green Olives)

Green to straw yellow olives are used. The olives are lye treated to destroy the bitterness, washed to remove the NaOH, and then brined for lactic acid fermentation. During washing, food-grade HCl can be added to neutralize the lye. Lye-treated olives are brined in 10–13% NaCl. Salt is added during the fermentation to maintain NaCl concentration at 5–6%. This can be increased to more than 7% at the end of fermentation to prevent growth of spoilage microorganisms. The optimum temperature is 24–27°C and fermentation proceeds for 3–4 weeks. The pH is 3.8–4.4 with 0.8–1.2% acidity after fermentation. Olives are packed into glass jars filled with 7% salt brine and sealed. The fermented green olives can be stuffed with strips of red pimento, small onions, and almonds. Pasteurization at 60°C or hot brine at 80–82°C can be used.

10.3.3.1.3 Untreated Naturally Ripe Black Olives (Greek-Style Olives)

Popular in Greece, Turkey, and North African countries, the olives used are completely ripened to purple or black before overripe. No lye treatment is used and thus the bitterness remains in the brine. These olives can have a fruity flavor and slightly bitter taste. Olives are put into tanks and covered with 6–10% brine. The mixed microbiota of coliform, yeasts, and Lactobacillus sp. are involved in the fermentation. The final acidity of the brine is less than 0.5% with a pH of 4.3–4.5.

10.3.3.2 Microorganisms in the Fermentation

The lye treatment of olives reduces initial populations of microorganisms and increases the initial pH to 7.5–8.5. Lye treatments and water washes remove sugars from the olives, reducing nutrients and available sugars. Olives are fermented by the same group of bacteria that ferment sauerkraut and pickles, Ln. mesenteroides, Lb. brevis, and Lb. plantarum. However, other species of Aerobacter, Escherichia, Bacillus, and yeasts remain longer in olives than in sauerkraut and pickles; thus, spoilage can easily occur.

Etchells et al. (1966) used pure-culture and mixed strains of Lb. plantarum, Lb. brevis, P. pentosaceus, and Ln. mesenteroides in heat-treated (74°C for 3 min) and unheated low-salt (5–6%) olives. Lb. plantarum dominated in the heat-treated, but not in unheated olives. Lb. plantarum LPCO10 isolated from green olive fermentation produced two bacteriocins. This bacteriocin-producing strain could predominate in olive fermentation and be used as a starter culture (Ruiz-Barba et al. 1994).

10.3.4 Kimchi

10.3.4.1 Processing

The baechu (Chinese cabbage) kimchi is the representative kimchi among 161 kinds of kimchi made in Korea. The preparation method is as follows (Cheigh and Park 1994; Park and Cheigh
The cabbage is trimmed, washed, brined (overnight in 10% brine), and then rinsed. The excess water is drained from the brined baechu cabbage (Cheigh and Park 1994). The pretreatment of the raw materials includes grading, washing, and cutting.

The premixture of chopped or sliced subingredients that typically include garlic, red pepper powder, salt-fermented fish (anchovy, etc.), and other vegetables are mixed and stuffed between the leaves of the cabbage (Figure 10.2). The standardized composition of baechu kimchi ingredients is as follows: the brined baechu cabbage (100%) is mixed with 13% sliced radish, 2% green onion, 3.5% red pepper powder, 1.4% garlic, 0.6% ginger, 2.2% fermented anchovy juice, and 1.0% sugar, and a final salt level of 2.5% (Cho 1999).

However, various other materials can also be added to the premixture of subingredients depending on family tradition, economics, and seasonal and regional availability of the materials. Watercress, mustard leaves, pear, apple, pine nut, chestnut, gingko nut, cereals, fishes, crabs, meats, and other ingredients, can all be incorporated into kimchi (Cheigh and Park 1994). The premixture mixed or stuffed cabbage is packaged and then fermented at different temperatures, but a low temperature (5°C) is ideal for preparing a good-tasting product.

### 10.3.4.2 Microorganisms in the Fermentation

*Lactococcus, Lactobacillus, Pediococcus, Leuconostoc,* and *Weissella* spp. have been isolated from kimchi. Choi et al. (2002) identified the genus *Weissella* from kimchi in the form of the novel species *Weissella kimchii*. The LAB profile during kimchi fermentation varies with pH and acidity. *Ln. mesenteroides* is observed during early fermentation (pH 5.64–4.27 and acidity 0.48–0.89%), and *Lb. sakei* becomes dominant later in the fermentation (pH 4.15 and acidity 0.98%) (Cho et al. 2009a). A distinct subset LAB related to kimchi fermentation is greatly influenced by temperature. *Lb. sakei* predominates in kimjang kimchi, and the strain appears suitable for low fermentation (5–9°C) and storage (–2°C) temperatures (Lee et al. 2008a). Ahn et al. (2003) isolated 27 bacterial strains from kimchi. They identified *Ln. mesenteroides, Ln. carnosum, Lb. curvatus, Lb. pentosus, W. kimchii, W. cibaria,* and *P. pentosaceus.* Most of the clones isolated from five commercially produced baechu kimchi were LAB (*Lactobacillus, Leuconostoc,* and *Weissella*).

**Figure 10.2 Preparation of baechu kimchi.**
Various starters for kimchi fermentation have been studied (Lee and Kim 1988; So et al. 1996; Kang et al. 1995; Chang and Chang 2010). Lee and Kim (1988) used the mixed strains of \textit{Lb. plantarum}, \textit{Lb. brevis}, \textit{P. cerevisiae}, and \textit{Ln. mesenteroides} isolated from kimchi. Adding the starters to kimchi shortened fermentation time, but also increased the flavor of the kimchi and showed consistent quality. Inoculating with psychotropic kimchi LAB starters resulted in sharp decreases in gram-negative bacteria and coliforms from the initial stage and also shortened fermentation time from 10 to 4 days at 8°C (So et al. 1996). The bacteriocin-producing strain, \textit{Ln. citreum} GJ7, from kimchi increased texture, sensory evaluations, and shelf life when it was used as a starter (Chang and Chang 2010).

### 10.3.5 Fermented Vegetable Juices

Fermented vegetable juices can be prepared by three different methods: spontaneous fermentation by natural microflora, fermentation by starter cultures added to raw materials, and fermentation by starter cultures added to heat-treated raw materials. The juices can also be pressed, in which juice is pasteurized and then inoculated with selected starter LAB cultures with $5 \times 10^6 – 1 \times 10^7$ CFU/g or CFU/ml (Karovicova and Kohajdova 2005).

\textit{Lb. plantarum} C3, \textit{Lb. casei} A4, and \textit{Lb. delbrueckii} D7 were used to produce probiotic cabbage juice (Yoon et al. 2006). Cabbage juice was inoculated with 24-h LAB cultures and incubated at 30°C. Cabbage, pH-adjusted tomato (7.2), carrot, red beet, and spinach were recommended as good vegetables for preparing vegetable juices since they have more fermentable sugars than other vegetables.

When starters are selected for the products, the following factors should be considered: the rate and total production of acids, change in pH, decrease of NO$_3$/NO$_2$ concentration and production of biogenic amines, ability of substrate to accept the starter culture, type of metabolism, and ability of the culture to create desirable sensory properties. Bacteriocin-producing starter cultures may be better for producing more controlled and reproducible vegetable fermentations. The health functionality of the fermented products is also an important factor for consumers.

The fermentation is usually performed at 20–30°C; however, lower temperatures should be considered to obtain better flavor and taste. During the fermentation, the pH of juices decreases from 6.0–6.5 to 3.8–4.5. A rapid decrease in pH in the beginning can minimize the spoilage. The most used probiotic bacteria are lactobacilli and bifidobacteria that can survive in the intestine.

Four LAB, \textit{Lb. acidophilus}, \textit{Lb. casei}, \textit{Lb. delbrueckii}, and \textit{Lb. plantarum}, were employed to develop red beet juice. \textit{Lb. acidophilus} and \textit{Lb. plantarum} produced more lactic acid than other cultures and reduced the pH of the beet juice from 6.3 to 4.5 after 2 days at 30°C (Yoon et al. 2005). \textit{Lb. plantarum} remained vigorously alive at the levels of $10^6–10^8$ CFU/ml when stored for 4 weeks at 4°C. Thus \textit{Lb. plantarum} from plants can be a good starter culture to develop functional probiotic vegetable juices.

### 10.4 Functionality of LAB from Fermented Vegetables

LAB from dairy products exhibit various functionalities and have been used as probiotics; however, few bacteria from fermented vegetables other than kimchi LAB have been studied for possible functionalities. Zeng et al. (2009) identified LAB with high conjugated linoleic acid (CLA)-producing ability from natural sauerkraut fermentation. The highest CLA-producing LAB was identified as \textit{Lb. plantarum}. The transformation efficiency of converting linoleic acid into
CLA was 26.7%. The *Lb. plantarum* produced a mixture of 32.2% *cis* 9, *trans* 11-c18:2 isomer and 67.8% *trans* 10, *cis* 12-C18:2 isomer, which may be responsible for several of the various functionalities of kimchi LAB.

### 10.4.1 Antimicrobial Activities of Kimchi LAB

LAB can produce organic acids, including lactic acid, during vegetable fermentation. They also produce CO₂, ethanol, diacetyl, H₂O₂, and bacteriocins, all of which can have antimicrobial activity. Kimchi LAB might also exhibit probiotic activities. Some of the probiotic mechanisms may involve modifying gut pH, antagonizing pathogens, and stimulating immunomodulatory cells.

#### 10.4.1.1 Antibacterial and Antifungal Activities

*L. mesenteroides*, *L. carnosum*, *Lb. curvatus*, *Lb. pentosus*, *W. kimchii*, *W. cibaria*, and *P. pentosaceus* were isolated and identified from kimchi. They showed variable antimicrobial activity against *Bacillus subtilis*, *Escherichia coli*, *Salmonella enteritidis*, *Sal. paratyphica*, *Sal. typhi*, *Staphylococcus aureus*, *Shigella boydii*, and *Sh. sonnei* depending on different stains (Ahn et al. 2003). The five LAB from kimchi, *Lb. curvatus*, *Lb. lactis* subsp. *lactis*, *Lb. casei*, *Lb. pentosus*, and *Lb. sakei*, showed a wide range of antifungal activity against *A. flavus*, *Fusarium moniliforme*, *Penicillium commune*, and *Rhizopus oryzae*. *Lb. casei*, *Lc. lactis* subsp. *lactis*, and *Lb. pentosus* are active against the indicator fungus *Aspergillus fumigates* (Kim 2005). *Lb. plantarum* AF isolated from kimchi inhibited the growth of *A. flavus*, *A. fumigatus*, *A. petrakii*, *A. ochraceus*, *A. nidulans*, *Epicoccum nigrum*, and *Cladosporium gossypicola* in a dual-culture overlay assay. *Lb. plantarum* AF1 also showed antibacterial activity against various species of gram-positive and gram-negative bacteria. The antifungal activity was stable after autoclaving and proteolytic enzyme treatment, and the antifungal compounds had low molecular weights (Yang and Chang 2008).

#### 10.4.1.2 Bacteriocin Production

Kimchi LAB produce various bacteriocins with antimicrobial activities. *Lb. plantarum* KC21 was isolated and identified from kimchi. This strain showed acid and bile tolerance, adhesion properties to a human intestinal cell line, and strong bacteriocin activity against pathogenic bacteria and can be a probiotic bacteria (Lim and Im 2009). *Lc. lactis* subsp. *lactis* A164 isolated from kimchi can produce nisin-like bacteriocin. The bacteriocin produced by the strain A164 was active against closely related LAB and some food-borne pathogens, including *Staph. aureus*, *Lis. monocytogenes*, and *Sal. typhimurium* (Choi et al. 2000). *Lc. lactis* subsp. *lactis* H-559, isolated from kimchi, exhibited antibacterial activity and was active against pathogenic bacteria such as *Lis. monocytogenes* and *Staph. aureus*. The antimicrobial substance produced by the H-559 was inactivated by α-chymotrypsin and protease types IX and XIV, and was confirmed to be a bacteriocin (Lee et al. 1999).

*Lb. paraplantarum* C7 was isolated and identified from kimchi. The strain produced bacteriocin, paraplantaricin C7, that inhibited certain *Lactobacillus* strains, including *Lb. plantarum*, *Lb. pentosus*, *Lb. delbrueckii* subsp. *lactis*, and *E. faecalis* (Lee et al. 2007). *P. pentosaceus* K23-2 isolated from kimchi produces a bacteriocin, pediocin K23-2, which is heat stable and shows broad-spectrum antimicrobial activity against gram-positive bacteria, especially *Lis. monocytogenes* (Shin et al. 2008).
10.4.1.3 Anti-Helicobacter pylori Effect

Helicobacter pylori is unable to colonize the stomach of Lb. salivarius infected gnotobiotic Balb/c mice; however, it could be colonized well, and cause gastritis, in germ-free mice. Lb. salivarius administered after H. pylori implantation could also eliminate colonization by H. pylori (Kabir et al. 1997).

Lb. plantarum NO1 isolated from kimchi showed strong antagonistic activity against H. pylori KCCM41756. The culture medium (2–4 μg/ml) of the Lb. plantarum reduced the urease activity of H. pylori by 40–60%. Lb. plantarum inhibited H. pylori binding activity to AGS human gastric cancer cells by more than 33%. Lb. plantarum showed high viability in 0.05 M sodium phosphate buffer (pH 3.0) for 2 h in artificial gastric juice (Lee and Chang 2008).

Ki et al. (2010) isolated Lb. paraplantarum KNUC25 from overfermented kimchi. The strains exhibited a broad antimicrobial activity against gram-positive and gram-negative bacteria. The cell-free supernatants (CFS) of Lb. paraplantarum showed anti-H. pylori activities when tested using the disc agar diffusion method. Adherence of H. pylori ATCC43504 or SS1 to AGS gastric cells was reduced by about 70% after 30-min incubation with 30 μl of 15-fold concentrated CFS. It has been reported that garlic and red pepper, which are the main subingredients of kimchi, reduced H. pylori infections (Iimuro et al. 2002; Graham et al. 1999; Jones et al. 1997). Lb. plantarum and Lb. paraplatarum if introduced above with the kimchi ingredients might further reduce H. pylori infection. Further studies are needed to investigate the efficacy of kimchi LAB and kimchi for treating and/or preventing H. pylori infections.

10.4.2 Antimutagenic and Anticancer Effects of Kimchi LAB

10.4.2.1 Antimutagenic Effects

The kimchi LAB, Ln. mesenteroides, Lb. brevis, Lb. fermentum, and Lb. plantarum, suppressed mutations induced by 4-nitroquinoline-1-oxide (4-NQO), 2-amino-3,4-dimethylimidazo[4,5-f] quinoline (MeIQ), and 3-amino-1-methyl-5H-pyrido[4,3-b]indole (Trp-P2) as much as Lb. acidophilus from yogurt, regardless of their viability in Ames test and SOS chromotest (Son et al. 1998). The investigators found that the strong antimutagenic activity of the LAB was due to the cell wall fraction rather than the cell cytosol (Park et al. 1998). Kimchi Lb. plantarum KLAB21 culture supernatant showed antimutagenic effect against N-methyl-N′-nitro-N-nitrosoguanidine (MNNG) and 4-NQO in the Ames test using Sal. typhimurium TA100 and TA98 (Rhee and Park 2000). Three different glycoproteins were identified as the antimutagenic substances from the kimchi LAB (Rhee and Park 2001).

The results also showed that whether kimchi LAB were viable or not, their antimutagenic activities were still effective at least as much as those of dairy-fermenting LAB. The peptidoglycan (PG) from the cell walls of Lb. delbrueckii subsp. bulgaricus was the major compound that suppressed tumor formation (Bogdanov et al. 1975). Hosono et al. (1990) reported that LAB especially Ln. paramesenteroides strains R-62 and R-8, etc., and filtrates obtained from cell suspensions inhibited mutagenicities of various nitrosamines using Sal. typhimurium TA98.

10.4.2.2 Anticancer Effect

Four strains were selected from kimchi samples that showed resistance to biological barriers (acid and bile salts). Lb. acidophilus KFR1342 from the four strains were identified and revealed the best
probiotic characteristics. The cytoplasmic fraction of the KFR1342 showed strong antiproliferative effects on tumor cells. The LAB strain inhibited less than 10% of normal cell growth but reduced the proliferation of SNU-C4 human colon cancer cell growth by 38%. The KFR1342 strain doubled quinone reductase activity and increased immunostimulating activities, increasing NO and IL-1α production (Chang et al. 2010).

Heat-treated and lyophilized LAB were administrated to ICR mice treated with sarcoma-180 cells. Tumor formation in the \textit{Lb. plantarum}- and \textit{Ln. mesenteroides}-treated mice was significantly reduced by 57% and 39%, respectively. \textit{Lb. plantarum} and \textit{Ln. mesenteroides} were administrated using Lewis lung carcinoma in C57BL/6 mice, and the inhibition rates were 42% and 44%, respectively. \textit{Lb. casei} also showed higher antitumor activities in that experiment (Kim et al. 1991). In another study, ascites tumors were induced by sarcoma-180 cells in Balb/c mice. Kimchi ingredients, the mixture of microorganisms from kimchi, and kimchi \textit{Lb. plantarum} (cell lysate) were fed (40 mg/kg/day) to the mice for 30 days. As shown in Figure 10.3, the expected life span of the mice decreased in the placebo group (average expected life span, 21.4 days); however, ascites tumor formation was markedly reduced and the expected life span was extended by 60% (the average life span, 34.2 days) by the feeding of kimchi \textit{Lb. plantarum} (Shin et al. 1998). Therefore, feeding or intraperitoneal administration of LAB suppresses tumors implanted in rodents. Muramyl dipeptide of the cell fractions might stimulate macrophages and release superoxide anion and hydrogen peroxide, which can kill tumor and bacterial cells (Chang et al. 2010).

10.4.3 LAB Counts and Intestinal Bacterial Enzyme Activities in Colon

Consumption of kimchi containing LAB significantly increased levels of \textit{Lactobacillus} sp. and \textit{Leuconostoc} sp. in colon ($p < .05$). Human subjects who consumed 300 g/day of kimchi ($10^8$ CFU/g of LAB) for 2 weeks during the kimchi phase had significantly increased counts of fecal \textit{Lactobacillus} sp. and \textit{Leuconostoc} sp., and had a decreased fecal pH. As shown in Figure 10.4, the counts of \textit{Lactobacillus} sp. significantly increased during the kimchi phase and decreased during the control phase ($p = .0003$). \textit{Leuconostoc} sp. counts showed a similar trend as \textit{Lactobacillus} sp. ($p = .0004$) (Kil et al. 2004). LAB in kimchi could pass through the human stomach, remain
viable probably by existing inside of the tissue of the cabbage, and then colonize in the large intestine. *Lb. plantarum* KCTC 3099 from kimchi can be colonized very well in the colon almost as efficiently as *Lb. rhamnosus* GG (Lee 2005).

In the study by Kil et al. (2004), fecal $\beta$-glucosidase and $\beta$-glucuronidase activities and pH were significantly decreased when 300 g kimchi was consumed by subjects, compared with the controls who consumed 60 g of kimchi for 2 weeks, the minimum amount in their customary diets, in four consecutive phases. $\beta$-Glucosidase can form toxic aglycones from plant glycosides, and $\beta$-glucuronidase hydrolyzes glucuronic acid conjugates and increases the enterohepatic circulation of toxic compounds. Low pH inhibits the growth of many pathogens and reduces the intestinal absorption of potentially toxic compounds (Martineau and Laflamme 2002). Lee et al. (1996) also reported that kimchi consumption significantly increased the levels of *Lactobacillus* sp. and *Leuconostoc* sp., and that the enzyme levels of $\beta$-glucosidase and $\beta$-glucuronidase significantly decreased during kimchi consumption.

$\beta$-Glucuronidase, nitroreductase, and azoreductase are the main human intestinal bacterial enzymes that stimulate the conversion of precarcinogen to carcinogen (Goldin and Gorbach 1976). In Koreans and Germans eating kimchi and sauerkraut, $\beta$-glucuronidase and nitroreductase activities in the colon significantly decreased, and a significant decline in fecal pH was also observed (Oh et al. 1993). *Lb. plantarum* can colonize in the intestinal tract for long periods and *Lb. plantarum* can make short-chain fatty acids from dietary fibers of the vegetables, which can induce apoptosis of colon cancer cells (Bengmark 2001), and may result in a preventive effect against colon cancer.

### 10.4.4 Increased Immune Function

The cell wall fraction of *Lb. plantarum* from kimchi activated the phagocytic activity of macrophages in mice. The polysaccharides binding with phosphodiester bonds to the muramic acid in the cell wall exhibited immune activity (Chung 1993). *Lb. plantarum* PS-21 from kimchi stimulated proliferation of Peyer’s patch cells, and the cell wall fractions exhibited strong mitogenic activity compared with the soluble cytoplasmic fraction. The peptidoglycan (PG) fraction was an active mitogenic component when used in murine lymph nodes and spleen cell test systems. PG

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**Figure 10.4** Changes in counts of *Leuconostoc* sp. and *Lactobacillus* sp. in fecal samples upon kimchi intake. (Kil, J.H. et al., *J. Food Sci. Nutr.* 9, 161, 2004. With permission.)
also enhanced antibody production in lymph node cells, and production of cytokines TNF-α and IL-6 in RAW 264.7 macrophage cells (Lee et al. 2006). Cell lysates of *Lb. plantarum* from kimchi showed immunostimulatory effects when fed to Balb/c mice. It increased the proliferation rates of splenocytes and Peyer’s patch cells; increased production of NO by peritoneal macrophages; and increased the production of intestinal secretory IgA, TNF-α, and IL-2 in blood. It was observed that oral administration of the cell lysate enhanced enteric and systemic immune responses (Chae et al. 1998). Administration of a culture broth of *Lb. plantarum* from kimchi to mice increased phagocytosis of *Staph. aureus*. Muramyl dipeptide and its derivatives can stimulate the cell-mediated immune function. This compound can stimulate macrophages and produce superoxide anion and H$_2$O$_2$ and kill tumor cells. *Lb. plantarum* from kimchi increased the generation of NO, TNF-α, and IL-6 in RAW 264.7 macrophage cells. *Lb. plantarum* from kimchi especially played a very important role in the immunopotentiating activity (Hur et al. 2004).

### 10.4.5 Antiallergy Effect

Allergy is caused by an immune reaction that is out of proportion to the antigenic stimuli. The physiological outcome is inflammation. Chronic allergic responses include asthma and eczema. Probiotics can regulate the induction of atopic dermatitis probably due to the ability to strengthen the mucosal barrier and prevent the circulation of undigested food proteins into the periphery of susceptible individuals. Atopic dermatitis is a chronic relapsing inflammation and is associated with the hyperproduction of IgE. 1-Chloro-2,4-dinitro- benzene was used to treat the backs of NC/Nga mice for 2 weeks. The kimchi LAB (*Lb. plantarum* fragment)–treated group had significantly lower serum IgE and serum IL-4 and IL-5 levels compared with the control group. The probiotic supplementation modulated IgE levels and IL-4 and IL-5 production. This result indicated that *Lb. plantarum* exerted its inhibitory effect via IL-4 production and thus inhibited IgE production in the atopic NC/Nga mice model (Lee et al. 2008b).

Asthma is a hyper-responsive condition in the airways, showing accumulation of various inflammatory cells, increase of mucus production, release of certain Th2 cytokines, and increased IgE levels. Either heat-killed *Lb. plantarum* or *Lb. curvatus* from kimchi showed a suppressive effect on airway hyper-responsiveness via upregulation of IL-10 or Foxp3 expression in lamina propria cells. This indicated that the kimchi LAB exerted suppressive effects on allergic diseases of airway hyper-responsiveness by intestinal immune modulation–inducing regulatory cells. *Lb. plantarum* especially reduced airway hyper-responsiveness. This may be due to the reduction of Th2 cytokines and an enhancement of Th1 cytokines in the lung and the induction of Foxp3 in the *Lactobacillus*-exposed intestines (Hong et al. 2010).

### 10.4.6 Antiobesity Effect of Kimchi LAB

In a study with high-fat diet (HFD)-fed SD rats, kimchi LAB powder (KL) resulted in reduced body weight and exhibited lipid-lowering effects. Groups supplemented with 10% and 20% KL had 13% and 15% lowered body weights compared with those given HFD alone. However, visceral fats were significantly reduced by 42% and 48%, respectively. Plasma triglyceride, cholesterol, and low-density lipoprotein (LDL) levels were also significantly lower, and triglyceride and cholesterol were excreted in the feces by the KL group (Kwon et al. 2004). This indicated that KL may have a similar role to dietary fiber. Kimchi LAB, *Ln. kimchii, Ln. citreum*, and *Lb. plantarum*, were used to ferment soymilk. The fermented soymilk (10 μg/ml) significantly reduced the contents of cellular triglyceride and inhibited cell differentiation, as shown by Oil red O staining, in
preadipocyte 3T3-L1 cells. The kimchi LAB–fermented soymilk inhibited the PPARγ2 expression and CCAAT/enhancer binding protein-α, transcription factors of adipocyte differentiation. The fermented soymilk significantly reduced plasma LDL cholesterol in HFD-induced obesity in SD rats (Kim et al. 2008).

Sousa et al. (2008) reported that administration of Lactobacillus supernatants (LS) into the brains of rats resulted in weight loss without a decrease in food consumption. Leptin expression in neurons and peripheral adipose tissue increased at the same time. LS modulated the expression or release of other peptides involved in metabolism and body weight control (Sousa et al. 2008). Kimchi LAB plus kimchi raw materials and fermented products showed antiobesity effects. Especially, optimally ripened kimchi significantly decreased body fats and body weights of animals (Park and Rhee 2005) and humans (Ahn 2007).

### 10.4.7 Kimchi LAB Degrade NO₂ and Insecticide

Kimchi LAB removed nitrite during fermentation. All sodium nitrite was depleted at the initial incubation (1–2 days) at 30°C and 36°C; however, as temperature was lowered the depletion rate was decreased. The isolated Ln. mesenteroides from kimchi depleted less than 20% of sodium nitrite after 10 days at 5°C, 86–93% after 7 days at 15°C, and more than 90% after 5 days at 20°C, and 2 days at 30 and 36°C (Oh et al. 2004). Depletion of nitrite was high as the order of Lb. plantarum, Lb. sake, and Ln. mesenteroides identified in the kimchi increased at 15–30°C. Nitrite was well utilized by L. plantarum during growth at all temperatures studied (Oh et al. 1997).

Lb. plantarum used as a meat starter culture depleted 59–93% of 200 µg of nitrite per milliliter of ATP broth over 24 h at 30°C. The depletion of nitrite was due to acid production during growth and enzymatic depletion of NO₂. Six isolates were found to deplete NO₂ up to 100% within 24 h at 22°C owing to their enzymatic activities (Dodds and Collins-Thompson 1984). Lb. lactis TS4 showed nitrite reductase activity induced by the presence of NO₂ and was active under anaerobic conditions. Nitrite reductase activity was detected in the supernatant fluid after centrifugation of cell extract at 226,000 × g for 1 h (Dodds and Collins-Thompson 1985).

The organophosphorous insecticide, chlorpyrifos (CP) (30 mg/L), was degraded rapidly until day 3 (83.3%) and degraded completely by day 9 at 8°C during kimchi (yeulmu-mulkimchi) fermentation (Cho et al. 2009b). Four CP-degrading LAB were isolated from the kimchi and identified as Ln. mesenteroides WCP907, Lb. sakei WCP902, Lb. plantarum WCP931, and Lb. sakei WCP 904. CP can be utilized by the strains as the sole source of carbon and phosphorus.

### 10.4.8 Other Physiological Functions of Kimchi LAB

The antioxidant activity of Lb. plantarum KCTC3099 from kimchi was evaluated by measuring the resistance to reactive oxygen species. Intact cell and cell-free extracts of Lb. plantarum showed higher antioxidative activity in inhibiting lipid peroxidation. The KCTC3099 strain could survive even after 8 h in the presence of both 1 mM H₂O₂ and 0.4 mM hydroxyl radicals, and in the presence of superoxide anion generated by using pararquat (Lee et al. 2005).

Lb. plantarum KCTC3928 from kimchi showed hypocholesterolemic effects in C57BL/6 mice. The doubly coated Lb. plantarum was fed along with an HFD to the mice. LDL cholesterol and plasma triglyceride levels decreased in live bacteria–fed group by 42% and 32%, respectively. The fecal bile acid excretion was increased by 45%. The live Lb. plantarum showed hypocholesterolemic effects in mice due to induction of fecal bile acid secretion followed by increased degradation of hepatic cholesterol into bile acids (Jeun et al. 2010).
Kimchi LAB, *Lb. buchneri* MS, produced γ-aminobutyric acid (GABA). GABA is produced by the α-decarboxylation of L-glutamic acid catalyzed by glutamate decarboxylase. GABA has hypotensive and diuretic effects, effectively prevents diabetes, and regulates neurological disorders. Certain levels of GABA need to be maintained in the brain to prevent epilepsy, seizures, convulsions, Huntington’s disease, and other diseases. The optimum condition for GABA production by the strain is provided by MRS broth containing 5% MSG, 1% NaCl, and glucose with an initial pH of 5.0 at 30°C for 36 h. *Lb. buchneri* produced 251 mM GABA with a 94% conversion rate. *Lb. buchneri* MS showed neuroprotective effects against the neuronal cell death induced by H₂O₂, retonone, sodium nitroprusside, paraquat, dieldrin, or MnCl₂ (Cho et al. 2007).

### 10.4.9 Functionality of Plant-Derived LAB

Sugiyama (2009) indicated that plant-derived LAB are superior to animal-derived LAB due to their acid tolerance, immune-stimulating activity, and intestinal regulation. Some strains of plant LAB produced anti-*H. pylori* substances when cultured in some fruit juice. *P. pentosaceus* LP 28 (LP28) decreased the amount of subcutaneous fat, and hepatic triglyceride and cholesterol levels in a mouse model of diet-induced obesity. Obesity-related genes, as determined by reverse transcription-PCR, were repressed by oral intake of the LP28 strain. Jin et al. (2010) indicated that the plant-derived LAB (*Lb. plantarum* SN13T, SN35N and *Lb. brevis* 925A), were more viable in artificial gastric fluid and bile than the animal-derived LAB (*Lc. lactis*, *Lb. bulgaricus*, and *Lb. acidophilus*). It was reported that yogurt prepared with plant-derived *Lb. plantarum* SN13T and SN35N (98:2) exhibited superior probiotic effects for improving constipation, serum lipids, and liver function as compared with the yogurt made with animal-derived LAB *Lc. lactis* A6, *S. thermophilus* 510, and *Lb. bulgaricus* C6 (86.1:13.8:0.1) in human trials (Higashikawa et al. 2010).

### 10.5 Functionalities of Kimchi

Kimchi (baechu kimchi) is a low-calorie food (18 kcal/100 g) and contains high levels of vitamins (vitamin C, β-carotene, vitamin B complex, etc.), minerals (Na, Ca, K, Fe, P), dietary fiber (24% on a dry basis), and other functional components such as capsaicin, allyl compounds, gingerol, isothiocyanate, and chlorophyll. Phytochemicals such as benzyl isothiocyanate, indole compounds, thioyuanate, and β-sitosterol are the active compounds found in kimchi that have shown antimicrobial, anticancer, and anti-atherosclerotic functions (Park and Rhee 2005).

Kimchi is mainly prepared with yellow-green vegetables, which have been claimed to prevent cancer, increase immune function, retard the aging process, and prevent constipation (Park 1995). When kimchi is fermented, its taste is enhanced and it becomes a good probiotic food. Table 10.1 shows the health benefits of kimchi that have thus far been documented. Kimchi showed antiobesity effects *in vitro*, *in vivo* (Park and Rhee 2005), and in human trials (Ahn 2007). Kimchi might prevent constipation and colon cancer owing to the high content of dietary fibers, organic acids, functional phytochemicals, and LAB.

Kimchi intake reduces serum cholesterol and increases fibrinolytic activity (Park and Rhee 2005), and thus has an anti-atherosclerotic function. Kimchi might retard the aging processes and delay skin aging (Ryu et al. 1997) owing to the antioxidative activities of vitamin C, β-carotene, phenolic compounds, and chlorophyll.

Table 10.2 shows kimchi ingredients (KI); with different NaCl content and fermentation methods, kimchi can exert different effects on the growth of MG-63 human osteosarcoma cells.
KI themselves show a low inhibition rate against proliferation of cancer cells. NaCl content from 1% to 5% did not affect the inhibition rate; however, kimchi fermentation with KI and 3% NaCl increased the inhibitory effect on the growth of the cancer cells. The kimchi fermented for 3 weeks at 5°C, which is an optimally ripened kimchi, inhibited the growth of the cancer cells by 3-fold. Thus, kimchi fermentation by LAB significantly increased the functionality of the products compared with the KI (fresh kimchi without fermentation) (Park 1995).

Figure 10.5 also shows that the optimum ripening of kimchi significantly induced apoptosis (expression of Bcl-2 family members) of HT-29 human colon carcinoma cells. It upregulated the expression of Bax mRNA and Bax protein as revealed by Western blot analysis; however, it down-regulated the expression of Bcl2 mRNA and protein. The detailed functionalities of kimchi had been already reported (Park and Rhee 2005; Kim 2009).

Table 10.2  Growth-Inhibitory Effect of Juice Supernatants of Kimchi Ingredients and Kimchi in MG-63 Human Osteosarcoma Cells after 6 Days of Incubation at 37°C

<table>
<thead>
<tr>
<th>Sample Juice Supernatant</th>
<th>200 µl/well</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>68 ± 10</td>
</tr>
<tr>
<td>KI + 0% NaCl</td>
<td>57 ± 7 (13)c</td>
</tr>
<tr>
<td>KI + 1% NaCl</td>
<td>54 ± 5 (20)</td>
</tr>
<tr>
<td>KI + 3% NaCl</td>
<td>52 ± 5 (23)</td>
</tr>
<tr>
<td>KI + 5% NaCl</td>
<td>49 ± 5 (28)</td>
</tr>
<tr>
<td>Kimchi (3 wk)b</td>
<td>23 ± 3 (66)</td>
</tr>
<tr>
<td>Kimchi (6 wk)b</td>
<td>30 ± 4 (56)</td>
</tr>
</tbody>
</table>


a KI: Kimchi ingredients (100% baechu cabbage, 2% red pepper powder, 2% crushed garlic, and 0.5 crushed ginger).
b Kimchi (3% NaCl) was fermented at 5°C for 3 or 6 weeks.
c Inhibition rate.
Possible Negative Effects of Fermented Vegetables

10.6.1 \( \text{NO}_3, \text{NO}_2, \text{and Nitrosamines} \)

Fermented foods generally have a very good safety record even in the developing world, although they are often produced in unhygienic and contaminated environments. Vegetables contain high levels of \( \text{NO}_3 \). Baechu cabbage contains 55–2500 ppm of \( \text{NO}_3 \) and 0.13–0.15 ppm of \( \text{NO}_2 \). Ten kinds of kimchi contained 0.25–0.68 ppm of \( \text{NO}_2 \), but nitrosamines (NA) could not be detected. However, contamination by \( \text{NO}_3 \), \( \text{NO}_2 \), and NA in kimchi was suspected. The level of \( \text{NO}_3 \) during fermentation was reduced from 292–300 ppm to 102–139 ppm after 6 weeks fermentation at 5°C, and the \( \text{NO}_2 \) content of the kimchi was 0.1–0.5 ppm after 6 weeks of fermentation. \( N \)-nitrosodimethylamine (NDMA) was a major NA found in kimchi, but the levels were not detected or were found in only trace amounts (0.044 ppb) (Park and Cheigh 1992). Kimchi showed

Figure 10.5 Effects of kimchi on protein and mRNA expression of Bax and Bcl-2 in HT-29 human colon carcinoma cells. (a) Protein expression of Bax and Bcl-2 in HT-29 cells. Actin band is shown to confirm equal loading of protein. Graph, fold ratio of protein expression. (b) mRNA expression of Bax and Bcl-2 in HT-29 cells. GAPDH band is shown to confirm equal loading of RNA. Graph, fold ratio of mRNA expression. (Kim, B.K. Antiaging effects and anticancer mechanisms of kimchi during fermentation. Pusan National University, Ph.D. dissertation, 2009. With permission.)
antimutagenic effects (Park et al. 1995) even with the trace amount of NA. Thus, kimchi has safe amounts of NO₃, NO₂, and NA contamination after the fermentation (Park and Cheigh 1992).

NA were not formed when the cabbage was supplemented with 250 or 1250 ppm of NO₃ nitrogen before the fermentation. Only one sample from triplicates formed 0.14 ppm of N-NDMA when 100 ppm of dimethylamine plus 100 ppm of NaNO₃ reacted in the freshly fermented cabbage for 48 h at 25°C. Thus, it is also concluded that NA are unlikely to be formed even if sauerkraut is prepared from NO₃-rich cabbage (Tate III and Alexander 1975).

*Lb. plantarum* 92H completely removed NO₃, and other starters had similar effects, for example, *Lb. plantarum* 90H (83% decline) and *Lb. delbrueckii* 37 (73% decline) during cabbage and carrot (1:1) juice fermentation. Their initial and final concentrations of NO₂ were only in trace amounts. It appears there is no danger of NO₃ and NO₂ contamination in LAB-fermented vegetables (Hybenova et al. 1995).

### 10.6.2 Salt

High intakes of NaCl may cause stomach cancer and high blood pressure (Takahashi et al. 1983). Salt itself did not show carcinogenicity; however, it can be comutagenic and cocarcinogenic when mutagens/carcinogens are present together (Kim et al. 1995). Comutagenicity of NaCl was remarkable especially with the direct mutagen, MNNG. The comutagenic effect of NaCl increased significantly (*p* < .05) as the NaCl concentration increased with MNNG. The revertant numbers were about three times higher when 15% NaCl was added to the MNNG in the system compared with MNNG alone (Kim et al. 1995). However, vitamin C decreased the comutagenicity of NaCl with MNNG-induced mutagenicity. High salt content (9.5–10.5%) in kimchi showed comutagenic effects, although the more typical kimchi that contained 3% NaCl showed antimutagenic activity (Park 1995).

The effects of salt in raw anchovy, salted anchovy (20% salt), and 6 and 12 months fermented anchovy (20% salt) on somatic mutagenicity induced by MNNG were evaluated by the Drosophila wing spot test system (Lee et al. 2000). Salt with MNNG increased mutagenicity and the frequency of large mwh spots from chromosomal recombination. No mutagenic activities were observed from raw, salted, and fermented anchovy samples in the absence of MNNG. The raw and fermented anchovy samples inhibited the mutant clone frequency of *Drosophila melanogaster* induced by MNNG, whereas salted anchovy increased the number of total mwh spots. The fermented anchovy juice extract showed antimutagenic effects. The fermented anchovy showed antimutagenic effects even though salt and salted anchovy without fermentation exhibited comutagenicity with MNNG.

Salt is needed for LAB fermentation of vegetables, and it seemed that the comutagenicity of the salt with raw materials was converted into antimutagenicity after the fermentation; however, we tried to reduce the sodium content by using different kinds of salt (Han et al. 2009) and fermentation methods, such as using beneficial starters. All of these are important factors for maintaining flavor and texture, but also for safety and functionality.

### 10.6.3 Biogenic Amines

Biogenic amines are biologically active natural compounds mainly from bacterial decarboxylation of amino acids. The monoamines histamine, tyramine, and tryptamine, and the diamines putrescine and cadaverine are formed from histidine, tyrosine, tryptophan, ornithine, and lysine, respectively. Putrescine is a precursor of polyamines spermidine and spermine (Halasz et al. 1994).
Excessive intake of biogenic amines in food causes psychoactive, vasoactive, and tumor growth effects (Kalac et al. 2000). The toxic dose strongly depends on the efficiency of the detoxification mechanisms of different individuals and other bioactive components in the fermented foods that protect against the toxic properties of biogenic amines.

In eight commercial sauerkraut samples, biogenic amines, especially putrescine, accumulated in sauerkraut brine: 100–200 mg/kg of putrescine and 42–52 mg/kg of histamine were detected in the sauerkraut, whereas 442–678 mg/L of putrescine and 143–174 mg/L of histamine were found in the brine. *Lb. curvatus* 2775 did not show any histamine synthesis and resulted in low total amine values on MRS medium. *Lb. plantarum* was present in small amounts, but was also a low amine producer (Halasz et al. 1999).

Cho et al. (2006) reported that kimchi contained 69.7 mg/kg of putrescine, 50 mg/kg of histamine, and 49.4 mg/kg tyramine. Mah et al. (2004) also determined biogenic amines from commercially prepared kimchi. Baechu kimchi contained 11.2–89.0 mg/kg putrescine, 0–5.1 mg/kg histamine, and 0–28.2 mg/kg tyramine. Biogenic contents were low in immature (8.6 mg/kg) and optimally ripened kimchi (11.7 mg/kg); however, putrescine levels (146.3 mg/kg) notably, and significantly, increased in overfermented kimchi. Using *Lb. plantarum* as a starter culture with $5.0 \times 10^{5–6}$ CFU/g significantly decreased putrescine, cadaverine, and tyramine contents compared with spontaneous fermentation. *Lb. plantarum* could depress proteolysis (Spicka et al. 2002). It is important to keep biogenic amine contamination to a minimum using fermentation methods; it is especially important to select appropriate starter strains that do not have biogenic amine–producing activity.

### 10.7 Conclusions

LAB in vegetables play important roles in promoting fermentation and in promoting human health by providing a source of probiotics. LAB identified from fermented vegetables or plant-derived LAB can be used as starter cultures for vegetable fermentation and fermented vegetable juices to provide a constant and better quality as well as functional properties. The various starters have different characteristics, and appropriate starters could be used for specific purposes when fermenting vegetable products. Vegetables contain various nutraceuticals along with natural LAB. Vegetables fermented by LAB produce various fermented end products, flavors, and functional bioactive compounds. Kimchi LAB and kimchi have been studied for their health functionality along with assessments of increased shelf life and improved taste of the product. Cabbage, cucumber, olives, and other vegetables also contain various phytochemicals that have health benefits. Vegetable products that are fermented with health-promoting LAB starter cultures will contribute greater health benefits than raw vegetables alone. The regulated fermentation technology might also increase health functionality, improve taste, and increase shelf life of the high-added-value products. However, some of the safety aspects, such as the high salt content of the products and biogenic amine formation in fermented vegetables, should also be considered and evaluated.

### References


Lactic Acid Bacteria in Vegetable Fermentations


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Chapter 11

Current Challenges for Probiotics in Food

Jean-Michel Antoine

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11.1 Introduction

Probiotics have been part of the traditional diet of many people for ages. Many legends have
promoted the benefits of specific fermented milks, claiming they were a source of well-being and
prolonged youth. Fortunately today physiologists and nutritionists have modern tools to decipher
what can be scientifically confirmed and what remains as hypothesis or fancy legends. At the same
time, a new “organ,” the gut microbiota, was discovered and scientists are studying its functions.
It provides a canvas for probiotics as they can mimic, support, or replace part of these functions
directly or indirectly. Another specific challenge for probiotics is to be a food that can please dif-
ferent tastes and be a part of many different balanced diets worldwide.
11.2 History of Use; First Challenge for Lactic Acid Bacteria: To Be Included in the Diet

Milk, the only substance designed by nature specifically to be a food, is a great medium for bacteria to grow, and recent data imply that some bacteria are able to develop in milk even at the source of production (i.e., in the mammary gland) (Martín et al. 2003). This also explains why raw milk is difficult to store: the environment is full of microbes that can grow in milk and spoil it. This is a challenge that humans had faced for ages until pasteurization was discovered. Still today the challenge is to select bacteria in milk that humans can tolerate, and maturation is part of many fermentation processes. One way to select those bacteria is to inoculate them in the milk as soon as possible; fermentation, a controlled use of microbes to preserve foods, is a very old trick humans have been using to prevent microbial spoilage of milk since the ancient times. How did the first controlled fermentation start? Nobody knows. It may have occurred by chance alone, or was the result of either a wise pragmatic observation or a long history of trials and errors, or even an adaptation of consumers to the most frequent microorganisms found in milk.

Milk has been for a long time collected in jars and tanks. It is easy to imagine that some of the microbes growing in milk were able to adhere to the walls of collecting jars and were selected due to the daily use of the same jars, resisting water cleaning if any. Those first-in-place and fastest-growing microbes were able to become the dominant species when fresh milk was newly poured in the jar. Subsequently they were able to establish a domination of the first in place and the fastest-growing microorganism.

Among those microbes, some were detrimental to human health and were called pathogens. Some were not, or humans became adapted to them, and they were called cultures. Humans possibly learned to recognize the smell and taste of some of the “pathogens” and differentiate them from the “cultures,” or selected the safe cultures according to their characteristic tastes and carefully used them for preservation, which became the traditional way of preserving milk.

Among these cultures, the lactic acid bacteria (LAB) are strong competitors because milk is a great ecological niche for them to grow: LAB can use lactose as a source of energy, and they are oxygen tolerant enough to survive transfers from pots to jar and even tanks in modern factories. They can develop specific systems to enhance their capacity to adhere to the jars, and some secrete sticky exopolysaccharides. They can also oppose some other microbial contaminants and competitors for colonization of that biotope. Furthermore, they are able to provide flavors and texture, and generate some easily recognized taste signatures that human taste buds can detect during ingestion.

This may explain why they are the most common microbes used for fermenting milks, and why fermented milks are part of the dietary culture of many tribes around the world. The acidity and the slight sourness of those fermented milks became part of our gustatory repertoire and we have learned to appreciate that taste, or those who favored that taste acquired a significant competitive advantage, either a nutritional one (being able to include dairy products in their diet) or a healthy one (being better protected than the non-users).

A picture of such a milking process can be found on a Sumerian stone (Figure 11.1), which shows the use of different jars where cultures can grow easily and be selected.

The same fermentation principle has been used by numerous groups of people, in many countries and at different times, with different milks and different microbial and physical environments. This ended up in a large variety of products and processes from the long fermentation occurring in cold caves in Norway to produce a kjaedermilk, to the daily shaking that promote fermentation of yogurts in a bag made of gastric stomach and warming up in the sun outside the door in Mongolia, including the domestic yogurt my mother was preparing every evening in eight
small glasses filled with boiled milk. We had to wait until the temperature was down to 45°C, using a specific thermometer, before she inoculated each glass with a spoon of the former yogurt. The eight glasses were kept on the radiator for the night, and the next morning we had fresh delicious yogurt.

There is a huge diversity of LAB and fermented milks around the world. This represents a nearly endless collection that modern scientists can dig into, to select the best cultures. The challenge is to understand why those cultures have been traditionally used, and what was the specific process to get the best of those traditional cultures and how to use them in the modern processes. Part of that challenge is that most of the time those cultures are not made of a pure single strain but are often a mixture of many different strains, when the modern fermentation aims at using a few single strains to ensure a good reproducibility of the process. It is fascinating to consider that for ages, and literally every day someone has been using one of these strains or cultures to make a fermented milk according to a traditional process to feed a group with that fermented milk, then store the culture for the next fermentation and produce another serving of fermented milk, all the year round, every year, to maintain the survival of that culture when milk is drying up, and finally to transfer that culture and its recipe to the next generation. The benefit of such a product and process must have been strong enough to trigger and support or reward for such a long-term investment. One of the best examples is the practice of sharing a grain of kefir and to give it to a son when he is leaving the family house. The advantage of a kefir grain is that it is visible without a microscope and it can be easily stored in dry conditions.

11.3 Challenge of Maintaining Fermentation Cultures and Reward of Fermented Milks

We are eating today in our yogurt, or some other fermented milks, cultures that have been used by many generations before us. It is still today one of the duties of producers and microbiologists collecting those cultures to maintain the vitality of the stored strains, and to “reanimate” them regularly from their frozen sleep: they are stored at −80°C and/or in liquid nitrogen, but they need some break and some fermentation cycles during their hibernation to maintain their vitality.

One of the challenges is to maintain the mother culture as stable as possible with a minimal number of duplications to reduce the risk of spontaneous gene modification that may occur during
any fermentation and growing phase. On the other hand, most of the traditional cultures have a very low frequency of spontaneous mutation, and this may be one reason they have been selected in the old times.

The taste signature of some cultures, and more specifically of some probiotics, may have played an additional role in the selection process. Humans use the taste of food as a first screening procedure, and we are all aware of that when someone ingest a new food and becomes sick in the following hours, the new food is considered to be the culprit, with its taste imprinted in the mind of the consumer, and will not be eaten again. In fact this is a very efficient system to prevent food poisoning (Garcia et al. 1955). The reverse is also true: there is a physiological reward system that helps us select the food we need, and it looks like we are prepared to learn to like the taste of foods that are generating some benefit after ingestion. The experimental demonstration has been done in rodents. When rodents are deficient in some specific essential amino acid, they are able to select a diet rich in particular amino acid and to learn the taste flagging that diet (Ashley and Anderson 1975). In humans this may be part of the explanation of the success of pasta parties organized the day before a marathon: runners learn to like the taste of this slow-release carbohydrate that will improve their muscular capacity in the following day. This may explain why some humans overcome the bitter taste of salicylate, which allowed the discovery of the use of willow bark to cure fever. It is interesting to note that the sour and acidic tastes of fermented milks do not commonly signal that a food will be liked. They are instead more often a warning signal: in fact, half of young children do not readily like the taste of fermented milks; they prefer the sweet taste of sugar (Schwartz et al. 2009). Therefore probiotics need to provide rewards strong enough to imprint a “good for life” feeling on the taste of probiotic foods, and to become part of many diets and be so popular. Many different traditions reported that some fermented milks were able to provide various health benefits to humans: In the Bible, according to Isaiah 7:22, yogurt was a reward: “everyone left in the land will eat yogurt and honey.” Abraham served yogurt to the three strangers (Genesis 18:8) visiting him and telling that he will have a son despite his old age, and the night after Sarah, his old wife, gave him a son!

In France it was reported that in 1542, King François the First was suffering from chronic diarrhea that no French physician was able to cure. His Majesty was aware of a Turkish remedy, yogurt, to cure diarrhea. In 1432, a French squire, Bertrand de la Broquière, made a trip to Jerusalem, and on his way back on foot he visited Turkey and discovered yogurt and mentioned it in his famous travel report to the king, Philippe le Bon. This report was part of the royal archives and must have been impressive enough to convince, one century later, François I to ask the “Great Turk” to send him some yogurt. A Turkish doctor went to France with his goat herd, made yogurt, and cured the king’s chronic diarrhea. This gave royal credit to the health benefit of yogurt, confirmed the report of de la Broquière, and was very frustrating for the French doctors.

Another legend is intriguing because it is found in many countries: It is commonly said that yogurt is milk that everyone can enjoy and digest. Milk contains a special sugar, lactose, which requires a specific enzyme, β-galactosidase, to be split into glucose and galactose that are absorbed in the small intestine. Most adults (actually all adult mammals) lose their β-galactosidase activity and suffer from lactose malabsorption and/or lactose intolerance when drinking milk. We will see that there are some modern scientific data to support the traditional belief of yogurt being an easy-to-digest milk.

Tradition is telling us that some fermented milks can provide added health benefits to the consumer, and their cultures were called “probiotics” or “good for life” to differentiate them from the technological cultures used to manufacture fermented milks (Fuller 1992).
The last legend was a challenging hypothesis, raised by a scientist in the early 1900s, Ilya Illich Metchnikoff. He was a Ukrainian physiologist working on immune defense mechanisms and he was awarded a Nobel Prize for his discovery of macrophages. He went to Paris to work in the Pasteur Institute, and he noticed that Parisians were dying at a younger age than Ukrainians. He did not look for a specific disease killing Parisians, although he was working with Pasteur, the microbe hunter; on the contrary he made the impressive hypothesis that there were some protective factors helping Ukrainians live longer than Parisians. At the end of his book on “prolongation of life,” he made the statement that the microbes of the gut may be manipulated either by food or by ingestion of “good bacteria” to replace the bad ones. In a way he was following the old tradition of Hippocrates, “let food be thou medicine.” This concept was formalized one century later by the definition of “probiotics” as “live micro-organisms which, when eaten in adequate amount, provide a health benefit to the host.”

11.4 Challenge of Probiotics; Diversity

All fermented milks have something in common: at the least they are all milk based, with an acidic pH resulting from the fermentation, and they contain a significant amount of selected microorganisms that help preserve the milk and reduce a risk factor for disease: microbial spoilage.

Fermentation changes the structure of milk proteins, reshaping the quaternary structure and hydrolyzing some amino acids or peptides from milk proteins. This will avoid the milk casein from clotting in the human stomach and improve milk digestibility by changing the two phases of gastric emptying of milk, with first a quick emptying of lactoserum, then later on the slow emptying of casein, into a homogeneous emptying of liquid (Gaudichon et al. 1994). This may be part of the legend saying that yogurt is milk anyone can digest.

Beyond these few commonalities, there are many differences. They belong to or are originating from a large diversity of microorganisms. The classification of probiotics includes different kinds of microorganisms:

- Some yeasts: *Saccharomyces kefir* is part of the core of Kefir, a very common fermented milk consumed in Eastern Europe; some *Kluveromyces* like in a Sudanese traditional fermented dairy product Rob; a specific strain of *Saccharomyces cerevisiae* (*boulardii)*.
- Molds such as *Aspergillus*, even if it is found in local fermented milk where it may be a contamination. More often proteases extracted from *Aspergillus* are used in some cheese processes.
- A lot of microbes. The most common genera used are *Lactobacillus*, *Lactococcus*, *Streptococcus*, *Leuconostocs*, *Pediococcus*, *Carnobacterium*, *Enterococcus*, *Vagococcus*, *Weisella*, and *Bifidobacterium*. In dairy products the most frequently used genera are *Lactobacillus*, *Lactococcus*, *Streptococcus*, and *Bifidobacterium*.

Each genus is split in numerous species: *Lactobacillus* is classified in 120 different species from *acetotoleraus* to *zymae*, through more commonly encountered species like *acidophilus*, *casei*, *delbrueckii*, *helveticus*, *plantarum*, *reuteri*, and *rhamnosus*. A species can be again separated into sub-species (e.g., *Lactobacillus delbrueckii* ssp. *bulgaricus*). Finally each species is made up of a large number of different strains with different numbers/codes according to the rule of the collection were they are stored. Classification is evolving, frontiers and names are changing with progress in knowledge and consensus. Thus, it is not always easy to trace back a strain in the bibliography.
Classifications have been based on phenotypes for a long time (e.g., sugar fermentation), and this was the basis of the so-called API system, or the use of a specific growth medium (e.g., for bifidobacteria). The first step required in this system is to be able to culture the strain in adequate conditions. This poses a challenge in the exploration of microorganisms.

Modern genetic tools are providing new rationale for classification, from the 16S RNA to global genetic maps, and they can identify microorganisms even when they are dead, owing to the conservation of DNA, or when we are currently not able to culture them. This will emphasize two points: first all strains have some specificities, and the functions they provide are more important than the name they were given.

### 11.5 Challenge of Probiotic Origin

Whatever the classification, consumers very often ask for the origin of a given strain, and they favor the claimed “human origin.” The confusion comes from the false intuitive concept that microorganisms belong to the place where they were first discovered; for example, a microbe collected in the feces of one human must originate from that person. In fact, microbes were living on earth before any mammals, and mammals are born sterile. They obtain their gut microbiota from the environment. All microbes found in the gut of any mammal, even humans, are coming from the outside, either acquired at birth or taken in through the diet. There are no probiotics, nor microbes, of human origin. Obviously when a microbe is already accustomed to a mother’s gut, it has a higher chance of colonizing the newborn and his or her gut because they are more likely to be part of the first “offer” from the environment. This may explain why some genera and species are more commonly detected in most human gut microbiota than others; however, they are not of human origin. The first microbe of the first mammal came from the environment, and this is still the case today. Therefore there is no probiotic of human origin, but there are probiotics able to appreciate human gut as a biotope where they can live transiently. However, during this long coevolution, microbes of the dominant human gut microbiota may have twisted their functions, improving those they need more in the human gut than outside and switching off and perhaps deleting those they do not use. They have developed a kind of “humanization.”

On the other hand, humans may have learned to rely on those common inhabitants of their gut and developed a symbiosis with microbes providing specific functions. It is worth mentioning that the dominant gut microbiota is harboring pathways to metabolize carbohydrates that humans are no longer able to metabolize.

Probiotics of fermented milks come from fermented milks, but they also first came from the environment. Any culture found in fermented milk has a former mother culture in the environment, as the cook has always a mother. It is a common practice to indicate where and when the probiotic was first collected, and to store it in an official collection.

There is no scientific answer to the question, “Where do probiotics come from?” This is not the least challenge from a consumer perspective. There is a very small proportion of probiotics among the huge world of microorganisms, and one of the challenges is to identify good candidates among that crowd. Many *in vitro* tests have been proposed to improve the selection of candidates to the probiotic status. The first rationale was to take the characteristics of the first probiotics as common golden rules to select other probiotics. The old books recommend analyzing the adherence capacity, or the resistance to bile salt, and some other criteria. Then it became evident that what was relevant for one probiotic and its related benefit was totally irrelevant for another probiotic and its different benefits. There is no, as far as I know, golden *in vitro* screening.
Current Challenges for Probiotics in Food

Probiotics are a diverse community. They can grow in very different conditions.

Optimal growth temperature: low temperature (10°C; 2–30°C) for psychrotrophic cultures, medium temperature (25°C; 5–60°C) for mesophilic cultures used mainly for cheeses, and high temperature (40°C; 30–65°C) for thermophilic cultures commonly used for fermented milks. Incidentally the local human gut temperature is around 37–38°C, where probiotics would have to work and generate a small local thermogenesis.

Length of fermentation: During fermentation, LAB produce lactic acid and lower the pH. Acidity is a self-limiting system that control fermentation, as LAB are sensitive to a too acidic pH. Therefore the kinetics of exposure to acid may change the internal metabolism, and a longer exposure to acidic conditions will decrease the internal buffering capacity of LAB and increase the production of heat shock proteins among other protective systems. A classical fermentation time for yogurt is 4–6 h, but the tradition in Mongolia is 1 day under the sun; some fermentations last for a few days; and there are long-term fermentations, at low temperature, for a few months.

Growth medium: Different cultures are used for different fermented milks, and different countries exhibit diversity by producing different milk varieties: Matsoni is made in the Caucasus from goat and/or sheep milk. Kumis is made from mare's milk and some specific kefir grains in Russia. Dahi is made from buffalo milk and sometimes fermented in bamboo tubes with a mixture of LAB in India. Also in India, Lassi is made from milk blended with sugar allowing some non-lactose-dependent bacteria to grow.

The importance of the growth medium can be illustrated by the following experiment where different strains were cultivated on two different growth media: milk on one hand and the classical de Man, Rogosa, and Sharpe (MRS) medium on the other. The marker of activity was the level of detoxification of a food carcinogen, IQ (2-amino-3-methylimidazo[4,5-f]quinoline), that is found in grilled meat. As shown in Figure 11.2, some strains were unable to detoxify IQ, some were able to detoxify IQ when grown on milk but not on MRS and vice versa, and some were able to detoxify IQ whatever the growing medium (Tavan et al. 2002).

One of the challenges in the study of the history of probiotics in food is to obtain a complete description of the cultures, the fermentation including the process, and the growing medium. This

Figure 11.2 Ability of different LAB strains to detoxify IQ depends on growing medium. (Modified after Tavan, E. et al., J. Dairy Res. 69, 335, 2002.)
was not common practice in old papers, and sometimes the only reference is the trade name of a product.

### 11.6 Challenge of Probiotic Survival

A common criterion for probiotics is the ability to resist digestive stresses. From the cup stored in the refrigerator to the human digestive tract, a probiotic in a food will face three challenges:

- A thermal one, from the storage temperature (-5°C), to the mouth where it will be warmed up to 25°C, before entering the stomach at 37–38°C where it will be stored for half an hour to a few hours, before entering the gut where it is supposed to work.
- An easy metabolic one as it enters into the mouth and the stomach coated with its dairy matrix. In the gut lumen it will encounter the digesta in a medium potentially rich in glucose, an easier source of energy than lactose. Furthermore, many other nutrients, including some peptides and amino acids before they are absorbed by enterocytes. It will not be too difficult to switch from a limiting acid dairy matrix to a warm nutrient-rich gut lumen.
- Last but not least, a chemical one with first an acidic challenge in the stomach: the fasting pH of an empty stomach is around 1 to 2, and then a quick neutralization back to pH 7.5 occurs, associated with detergent bile salts in the duodenum.

Probiotics ingested as fermented milks can cope with the gastric challenge in two ways:

First when fasting humans drink fermented milk, the luminal pH of the stomach becomes closely similar to the pH of the product (Figure 11.3). After ingestion of 250 ml of yogurt, the pH of the stomach of five human volunteers remained at around 4 for more than 1 h. The second protection is afforded by the buffering capacity of dairy proteins that are amphoteric. This may also help the probiotics support the stress when arriving in the duodenum where the gastric luminal pH is neutralized by biliary and pancreatic secretions to pH 7.5.

![Figure 11.3 Variation of pH in stomach of five adults after ingestion of 250 g of yogurt or 250 ml of milk. pH on vertical axis and time in minutes on horizontal axis. (Modified after Dorval, E.D. et al., *Bactéries lactiques*, VI.6, p. 420, Lorica ed., Uriage, 1994.)](image)
This neutralization will allow the probiotics to start metabolizing and potentially grow again without the previous inhibition of acidity. Then luminal detergent bile salts will challenge the organization of the cell membrane of dividing probiotics.

All strains are not equally able to cope with such challenges: within 22 strains of *Lactobacillus bulgaricus* fermenting in an exponential growing phase and exposed *in vitro* for 30 min to a pH of 3.5, one lost less than 1 log, one lost more than 5 log, and the others were in between. The same strains, in a second similar experiment but exposed to an experimental biliary stress using taurodesoxycholate, expressed similar sensitivity: one very resistant, one very sensitive, and the others in between (Guillouard et al. 2004). Unfortunately the most acid-resistant strain was not the most resistant to biliary stress and vice versa. However, there are a few strains able to cope fairly well with both stresses. They are potential candidates for the probiotic status, being able to survive the upper human digestive challenges. Furthermore, it has also been shown that most strains can be adapted to cope better with acidic stress if they are properly “trained” by successive fermentations in increasingly acidic conditions. This is due to the induction of heat shock protein (Hsp): the more Hsp, the greater the adaptability. Some lactobacilli were able to increase their resistance by more than 100-fold.

One of the challenges is to select a candidate with a fair resistance to both stresses as well as a significant ability to improve that resistance. It goes without saying that this is critical for the probiotic that needs to survive beyond the duodenum. In fact, one mechanism of action of some probiotics is to release internal enzymatic activities in the lumen. Probiotics that become active by releasing their enzymes in the upper part of the gut must be sensitive to those stresses, and the survival criteria are not valid for these specific probiotics.

This may become a specific challenge for those probiotics that will act as shuttle-delivering specific compounds at a given place in the gut. It may be impossible to select a probiotic that is active in the stomach because it will be killed by the local pH. The survival will then be limited to the transit to the stomach. This adaptability has been used to select and to adapt a specific strain of *Bifidobacterium lactis* DN 173 010 and to overcome the usual sensitivity of bifidobacteria to stomach acidity (Berrada et al. 1991). That strain is able to survive in the human gut and is recovered in the feces of those consuming it at the same concentration as in the serving: 10⁸ CFU/g.

Survival during the transit is only a transient colonization. Probiotics are part of the transient microbiota but are not part of the resident microbiota. One week after the last consumed serving, probiotics are usually no longer recovered in the stool of the above-mentioned consumer. Some strains are able to stay longer in the gut, but the rule is that the resident microbiota will not tolerate a foreigner to become part of a given individual microbiota. As for all foods, there is a need for regular consumption of a probiotic to benefit from it every day.

### 11.7 Challenge of Being Active

Surviving is important, but the next challenge for a probiotic will be to be able to be active in the human gut. Gérard Corthier used a molecular flag to explore the metabolic activity of a strain of *Lactobacillus casei* in a human microbiota rat model. He inserted a luciferase gene in the lactose operon to detect the activation of the lactose pathway. When *L. casei* switched on some metabolic activity, it had to switch on energy consumption and the lactose operon, including the luciferase gene. It is then easy to detect the production of light by the luciferase and to monitor the metabolic activity of *L. casei* in the gut. One and a half hour after ingestion, some light was detected in the
small intestine; that was 1 h after the first *L. casei* was detected in the small intestine (Oozer et al. 2004). A similar luciferase activity was detected in the colon at the same time, when *L. casei* arrived in the colon. This indicates that the strain of *L. casei* needed around 1 h to become active in the humanized gut of a gnotobiotic mouse. In a later study, it was shown that *L. casei* was activating only part of its machinery (Oozeer et al. 2005), while a strain of *Streptococcus thermophilus* was able to switch on its lactase activity in the small intestine (Mater et al. 2006).

One of the challenges of probiotics in foods is that they have to deal with two different biotopes: the first one is milk where they are growing and fermenting, and the second is a human gut where they have to deliver their benefits. Thus they should have two different metabolic behaviors, with the latter being the more important from a human perspective.

The most challenging part of studying probiotics is to decipher their mode of action and to implement a food production process that will not damage those mechanisms (Rabot et al. 2010). They can act directly in the human gut, thanks to the enzymatic activities they are providing. One of the most studied enzymatic activities is their ability to aid in the digestion of lactose. Fascinating enough, it can explain the most common legend about yogurt as being milk anyone can digest. Using the breath test methodology, Kolars et al. (1984) demonstrated that living yogurt cultures were able to digest the lactose eaten with the experimental serving of yogurt, as illustrated by the dramatic reduction of breath concentration of hydrogen, reflecting the decrease of lactose reaching the cecum. This effect has been confirmed by many different teams in different countries with different yogurt cultures, reporting a variable but always significant reduction of lactose malabsorption in lactose nondigesters who consume yogurt. Some of these studies reported that some probiotics from other species can also improve lactose digestibility, but far less than yogurt. It has also been demonstrated that one of the partners of the yogurt symbiosis, *L. delbrueckii* ssp. *bulgaricus*, was able to improve lactose digestibility without prior fermentation, but that the digestibility was significantly improved by fermentation, using the same number of microbes (Martini et al. 1994).

It is still not very clear what is the enzymatic activity involved in that improved lactose digestibility. The efficacy of the different strains of lactobacilli in the human gut as measured by the breath test is not proportional to their content in lactase activity per CFU, and it seems a permease activity, involved in the influx of lactose into the probiotic, may play a role (Savaiano and Levitt 1987).

A more conflicting observation: Improvement of lactose absorption is not totally correlated with improvement of clinical symptoms of lactose intolerance associated with lactose malabsorption. This suggests that there are two places of action: one in the small intestine associated with the digestion of lactose, and one in the colon associated with the control of luminal fermentation (Zhong et al. 2004).

### 11.8 Challenge of Dealing with Endogenous Microbiota

The gut has two parts: first, the small intestine with a low concentration of resident microbiota where $10^7$–$10^8$ probiotics per gram (the average concentration of probiotics in fermented milks) is a significant concentration and where they can provide a biologically significant concentration of enzymatic activities. Second, the colon with a high concentration of resident microbiota where $10^8$ probiotics per gram is minute compared with the resident colonic microbiota.

Another recently reported enzymatic activity that a probiotic can provide is the reduction of hyperoxaluria that *Oxalobacter formigenes* induces by degrading oxalate in the lumen of the gut, therefore reducing oxalate absorption, oxalate excretion in urine, and the risk of kidney stone development (Okombo et al. 2010).
A further kind of direct action is the secretion of antimicrobial factors: some probiotics secrete such compounds to prevent potential competitors to grow locally. This is named a barrier effect or microbial exclusion. This mechanism was involved in the selection of the first probiotic strain, *Escherichia coli* Nissle, in 1917, which was able to eradicate *Salmonella* from healthy carriers.

A second mode of action of probiotics is to interfere with the host microbiota, either by temporarily replacing a missing part of the resident microbiota, or by adding its transient concentration to the endogenous population, or by stimulating part of the resident microbiota. Whatever the mechanisms, a probiotic strain of *L. casei* increased the concentration of lactobacilli in the stool of young children (Guerin-Danan et al. 1998); a strain of *Lactococcus lactis* increased the concentration of bifidobacteria and reduced the concentration of enterococci (Bernbom et al. 2006).

There is a rising opportunity for future probiotics. In the last decades the role of microbial pathogens on digestive diseases has been demonstrated. The best example is the role of *Helicobacter pylori* in the pathogenicity of gastric ulcers. However, the unsuccessful quest for a pathogen for some other diseases such as Crohn’s disease has triggered the new hypothesis of missing microbe as the cause of disease, rediscovering Metchnikoff’s hypothesis, and opening a new potential for probiotics. The genomic analysis of the missing function will also help select the potential probiotic harboring that requested function.

Changes of the endogenous microbiota can also be monitored by analyzing changes in enzymatic activities; for example, nitrogen metabolism as reflected by the urinary concentration of p-cresol, the glucosidases, the biliary salt hydrolases, or azoreductase (Ouwehand et al. 2002). The list will increase in the coming years, expanding the potential of other probiotics.

One of the present challenges is to explore how some probiotics may modulate the efficacy of the gut microbiota to recover part of the energy of indigestible dietary components arriving in the colon, and modulate the global energy density of human diet.

Finally it has been recently demonstrated that some probiotics are able to either switch on or off some of the genes of gut cells, first on animal models, and subsequently in humans (Sonnenburg et al. 2006). It is fascinating to discover that microbial prokaryotes are cross-talking with our eukaryotic cells, reinforcing the concept that at the very beginning we may have had a common ancestor, and the archeabacteria found in the human gut may be the link to that ancestor.

This complexity in the mode of action should not be a surprise; it is not different from the complexity of the mode of action of our resident microbiota. Moreover, there is still one part of that world that is not easy to explore: the microbiota associated with the gut mucus.

### 11.9 Probiotic Challenge of Providing a Health Benefit to Humans

Overcoming this challenge must take into account the present limits of the scientific knowledge on the role of the gut microbiota, and the physiological variabilities among the human species. This may explain why many scientists are using diseases or borderline situations as models to explore the beneficial effects of potential probiotics.

The use of such models was the classical and historical approach in nutrition science, for example, the discovery of vitamins. The first observation came from the use of a food on patients suffering from severe deficiencies either on board a boat (for scurvy) or in a prison (for beriberi). Understanding the different physiological targets of vitamin C or B1 was a long process, and is still not completed to date for some vitamins, such as vitamin D.
Similarly for probiotics, the first results were reported on the efficacy of some probiotics against diarrhea, and modern science is exploring different physiological targets of probiotics, and to follow on with the comparison with vitamins. As under the name of vitamins there are very different compounds, within the category of probiotics there are many different strains with different benefits involving different mechanisms that modulate multiple functions or pathways.

A recent revolution has occurred; the exploration of the gut microbiota with new tools, overcoming the necessity to culture microbes, and the beginning of understanding the network of functions it is involved in. This adds some complexity to the central challenge. This forgotten and hidden organ is becoming a second body within our body, and Joshua Lederberg, recipient of the Nobel Prize in Physiology in 1958, referred to a supra-organism in which the microbiome will be the dominant partner (with 10 time more cells, 100 times more genes and functions) and our body a minor one. In fact, neither the exact role nor the physiology of this new organ is well understood. It is therefore challenging to explore a probiotic when part of its target is not well defined.

On the other hand, there is a vertiginous and challenging potential for probiotics to modulate a function; reinforce, transiently or not, a weak one; or even replace a missing or a destroyed one. This may happen after an antibiotic treatment or with age.

Solid, validated markers are lacking in this rapidly evolving field. Markers are a cornerstone for conducting convincing randomized controlled human trials and for deciphering the exact cause–effect relationship. However, the integrated global benefit of probiotics can be demonstrated without the understanding of the exact involved mechanisms.

This mechanistic part will become more and more important in exploring culture collections for the selection of future probiotics: it will be easier to look for a given function when it can be identified at the gene level. This complex field needs a rigorous approach, and simultaneously an open mind, to explore new rising opportunities with the help of modern exploratory tools.

It is impressive to read the exponential scientific literature on probiotics, reflecting the growing field of interest. Furthermore, many meta-analysis confirmed that (Szajewska 2010)

- Different probiotics generate different benefits.
- Some probiotics fulfill the definition and provide relevant health benefits to the host.

Once a probiotic has been identified, the second cornerstone challenge is to build a food around that probiotic; that is, a tasty, affordable, and reproducible food.

Most of the time different cultures are associated with aroma production, texture development, and ensuring a reproducible fermentation. These symbionts must be able to grow in the various milks that are entering factories all the year round, in different countries, and to deliver the same product day after day. This is shorter and easier to write than to do. Pilot tests are performed at different scales until the final end test in the factory. It is an iterative process that can take many years.

### 11.10 Final Challenge for Probiotics Exploring Safety

On one hand, humans have learned that there is no safe diet, and annual reports of food safety agencies confirmed the high incidence of food-borne diseases every year. On the other hand, fermented milks have been consumed for ages by different populations with different health conditions, and even with many diseases. Fermented milks have even been used to treat many different diseases. There is no traditional safety concern with the use of fermented milks; they are not part
of the reported food-borne diseases despite the large consumption, even when some food-borne diseases were related to milk consumption. However, probiotics are living microbes, and in pathological conditions, for example, when the normal propulsive ability of the gastrointestinal tract is disrupted, they may grow among the endogenous microbiota and participate in local acidification. They can also translocate, following the physiological pattern of the endogenous microbiota, although probiotics are among the safest members of the human gut microbiota. It cannot be excluded that some specific targeted probiotic may have potential side effects, reflecting the specificity of action. For example, modulation of inflammation will be prone to such benefit/risk analysis because of the complex balance of regulatory systems.

11.11 To Conclude

Probiotics in food are facing many challenges today:

- It is a recently rediscovered category of food with such a large variety of benefits that it is impossible to summarize the whole field in a simple, accurate way.
- There are more differences than commonalities between probiotics. Assessment of a probiotic is by a case-by-case analysis.
- There are many legends and traditional beliefs about probiotics, from being an easy-to-digest milk to being a panacea for long-term youth. Not the best start to convince scientists. Demonstration of a benefit is a long and costly exercise that may increase too much the prize of the probiotic food.
- There are no \textit{in vitro} tests able to predict which candidate will be a real probiotic with a demonstrated benefit in humans. The ultimate proof comes from randomized controlled human trials, and it is difficult to design convincing randomized controlled trials.
- What is the level of scientific demonstration needed to find the truth in old legends?
- What is the level of science needed before implementing a beneficial dairy probiotic in a diet?
- What is the level of science needed before offering a rationale to consumer to help them make a fair choice? Probiotics are partners of the gut microbiota, and the physiology of the gut microbiota is revisited. It looks like this ignored “organ” is a new world; a bio-equivalent to our body, or even bigger. Exploring this new “organ” is a rapidly evolving science, and consensus on relevant markers has not yet been achieved.
- They are food, providing moderate daily benefits, and lead the way for a positive improvement of our diet.
- Finally, food processing must take into account the constraints of the specificity of the probiotic, and deliver tasty food in a stable, reproducible way.

References


Chapter 12

Lactic Acid Bacteria in Cereal-Based Products

Hannu Salovaara and Michael Gänzle

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12.1 Summary

Cereal-based foods are a major source of inexpensive dietary energy and nutrients worldwide. Cereal grains readily support the growth of microorganisms, including lactic acid bacteria, provided water and hydrolytic enzymes are present. Certainly many cereal foods, such as boiled or steamed rice, porridge, pasta, and cookies, are made without any fermentation process, and when fermentation is used, alcoholic fermentation by yeast prevails, as in bread making and brewing. However, alcoholic fermentation of cereals often also involves lactic acid fermentation, and a mixed flora occurs. A number of cereal-based foods are characteristically fermented by lactic acid bacteria, such as the European rye and wheat breads, various Asian flat breads, and numerous types of fermented sour porridges, dumplings, and non- or low-alcoholic beers common in Africa, South America, and elsewhere. In these applications, alcoholic fermentation may also have a role, but the lactic acid bacteria contribute to the technological and nutritional benefits, as well
as influence the flavor and keeping properties of the products. Most fermented cereal-based foods are heat treated after fermentation, and the bacteria are killed. However, there are also cereal-based foods that contain live lactic acid bacteria, both traditional lactic-fermented foods and novel applications. This chapter discusses some aspects of the traditional and novel role of lactic acid bacteria in the production of cereal-based foods.

12.2 Cereals as a Substrate

Cereals are, in general, a good medium for microbial fermentations provided there is enough water available. The level of free sugars in matured sound grains may be only 1–3% (Table 12.1), but even this low amount supports the initiation of the fermentation process, and in many processes endogenous cereal enzymes will produce more sugars from the polysaccharides. In rye the contents of free sugars and amylolytic enzymes are higher than in other cereals, and this may partly explain the association of rye and sourdough baking. Endogenous cereal enzymes, added malt, or enzymes can be used to break down the starch to simple fermentable sugars.

Lactic acid bacteria capable of utilizing starch are also known and may be present in cereal fermentations (Nguyen et al. 2007). Besides carbohydrates, cereals also contain minerals, vitamins, sterols, and other growth factors, which support growth of microbes, including fastidious lactic acid bacteria. Cereal grains normally carry an indigenous microbial flora composed of a variety of different microbes, such as molds, enterobacteria, and aerobic spore formers, all of which compete for nutrients. Since no pasteurization can be generally applied without affecting the technological

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Content (% Dry Matter Basis)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polysaccharides (total)</td>
<td>70–80</td>
</tr>
<tr>
<td>Starch</td>
<td>45–77</td>
</tr>
<tr>
<td>Dietary fiber (as nonstarch polysaccharides in lignin)</td>
<td>9–15</td>
</tr>
<tr>
<td>Low molecular weight carbohydrates (total)</td>
<td>2–5</td>
</tr>
<tr>
<td>Fructose</td>
<td>0.1–0.4</td>
</tr>
<tr>
<td>Glucose</td>
<td>0.1–0.5</td>
</tr>
<tr>
<td>Sucrose</td>
<td>0.5–2</td>
</tr>
<tr>
<td>Raffinose</td>
<td>0.2–0.7</td>
</tr>
<tr>
<td>Protein</td>
<td>8–15</td>
</tr>
<tr>
<td>Lipids</td>
<td>2–6</td>
</tr>
<tr>
<td>Ash (minerals)</td>
<td>1.5–3</td>
</tr>
</tbody>
</table>

properties of starch and protein, a vigorous starter flora of lactic acid bacteria is required for a rapid lactic fermentation.

### 12.3 Lactic Acid Fermentation in Wet Milling

Dry cereal grains, being dry and hard nutritive packages, can be eaten only after milling or grinding and mixing with water. Hydrolysis by endogenous enzymes and fermentation by various microbes, including lactic acid bacteria, is often an unavoidable and integral part of the aqueous stage of cereal food preparation. For example, soaking of grains in water before wet milling gives rise to various fermentation processes, including the lactic acid fermentation that often prevails in the end. Soaking of grains in water before wet milling is customary when corn, sorghum, or millet is ground in traditional food processing. Soaking softens the grain endosperm and greatly reduces the work input required for grinding. Penetration of water into the interior of the kernels takes hours, and simultaneous fermentation occurs. The fermenting microorganisms originate from the surface of the kernels and from the steeping vessel and other equipment (Odunfa 1985; Wacher et al. 1993; Holzapfel 2002; Nout 2009). The resulting wet starchy material continues to undergo fermentation and carries the sour flavor, which is typical of indigenous foods cooked from fermented slurries (Campbell-Platt 1987; Hounhouigan et al. 1993).

Lactic acid fermentation also occurs and is utilized in the preparation of many tropical staple foods, a topic recently reviewed by Nout (2009). In these processes, the lactic acid bacteria not only enhance flavor and texture but also inhibit pathogenic and spoilage organisms by several mechanisms, such as the production of organic acids, hydrogen peroxide, and antimicrobial substances, as well as by lowering pH and oxidation/reduction potential (Mbugua and Njenga 1992). Lactic acid bacteria starter cultures have also been studied for controlling undesirable microbial growth in malting (Laitila et al. 2006).

When oats were prepared for food, one technique formerly used was the separation of hulls by slurrying stone-ground oats with water. The procedure enabled the hulls to be strained from the surface, whereas endosperm particles sedimented. The slurry underwent simultaneous sourdough-type fermentation, and this was favored by adding rye sourdough (Lampinen 1976). The sour starchy sediment was used to cook indigenous fermented porridges and gruels, such as the Welsh flummery and Karelian kiesa (Fenton 1974; Salovaara et al. 1991).

### 12.4 Lactic Acid Bacteria in Bread Making

#### 12.4.1 Use and Functions of Sourdough

Sourdough is fermented by a mixed flora of lactic acid bacteria as the numerically dominant microorganisms, and yeasts. Historically, dough leavening was the principal technological function of sourdough in baking. The use of sourdough as a leavening agent was widely replaced by baker’s yeast only after *Saccharomyces cerevisiae* was industrially produced as a leavening agent in the late 19th century (Pederson 1971). The availability of standardized and metabolically active baker’s yeast, and the substantial reduction of the time required for proofing favored the use of straight dough processes with baker’s yeast as the sole fermentation organism in wheat baking. Throughout the 20th century, the use of sourdough as a leavening agent in wheat products was limited to specialties, such as Panettone (Italy), specialty baguettes (France), or the San Francisco
sourdough bread (United States). In these products, sourdough not only serves as a leavening agent but is also indispensable to attain the typical appearance and flavor.

The gas-holding capacity of wheat dough is dependent on the properties of highly polymeric gluten proteins, which are absent in other cereals. In contrast, the gas-holding capacity of rye dough is dependent on polymeric arabinoxylans, which require solubilization during an extended incubation time for optimum technological functionality. This is one of the reasons why the use of sourdough is continued in rye baking and for production of various flat breads. Sourdough is increasingly used also as a baking improver in wheat and rye baking to enhance flavor, texture, and shelf life, and to replace additives. Today a substantial proportion of sourdough is not fermented in bakeries but supplied to the baking industry in dried or otherwise stabilized preparations. Approximately 30% of bread produced in Europe and an increasing proportion of bread produced in North America involves the use of sourdough or sourdough products.

Sourdough affects all aspects of bread quality, including bread texture, flavor, and shelf life. The benefits of lactic fermentation are apparent in all breads but particularly pronounced in rye baking. Excessive sourness in white wheat bread is considered an off-flavor by most consumers, whereas in rye bread sourness is a desirable flavor attribute and is favored over nonacidified rye bread in northern, central, and eastern Europe. Dough for the various flat breads made in parts of Asia and Africa is often fermented in a process resembling that used for sour rye bread in Europe. Sourdough is also used for non-wheat breads or cakes, such as injera and kisra flat breads made from sorghum and other local cereals in Ethiopia and Sudan. Indian idli, made from rice and black gram, and puto, another steamed rice cake used as a snack in the Philippines, are also examples of breads or cakes made with sourdough (Food and Agriculture Organization [FAO] 1999; Nout 2009).

The functions of sourdoughs in bread making are listed in Table 12.2. Dough leavening by formation of carbon dioxide is achieved by the combined activity of yeasts and heterofermentative lactic acid bacteria (Unbehend and Brümmer 1998; Hammes and Gänzle 1998; Häggman and Salovaara 2008a). In an Indian rice/legume-based aerated cake-type food, idli, heterofermentative lactic acid bacteria (Leuconostoc) were reported to be the microorganisms responsible for leavening (Mukherjee et al. 1965). There is also some consumer interest in traditional bread making without added baker’s yeast (Unbehend and Brümmer 1998; Häggman and Salovaara 2008b).

The technological benefits of sourdough procedures in traditional rye bread making also include the suppression of high endogenous activity of α-amylase and the conversion of insoluble pentosans to soluble polymers to improve the water-holding capacity and the gas retention (Röcken and Voisey 1995; Hammes and Gänzle 1998). Solubilization of arabinoxylans during fermentation also occurs in wheat sourdoughs, but in wheat doughs arabinoxylans are only of minor importance for dough hydration and gas retention in comparison with gluten. Exopolysaccharides produced by lactic acid bacteria during baking also act as water-binding hydrocolloids to improve bread volume and texture (Gänzle and Tieking 2005; Arendt et al. 2007; Katina et al. 2009). Amylolytic strains of lactobacilli were used in sourdoughs to inhibit staling by decreasing retrogradation through the enzymatic modification of starch (Corsetti et al. 2000). A majority of lactobacilli do not possess extracellular amylases; however, amylolytic lactobacilli were isolated particularly from fermentations of cereals with low endogenous amylase activity (i.e., sorghum, maize, and cassava) (Gänzle and Schwab 2009). Modification and partial hydrolysis of proteins in wheat and rye sourdoughs also occurs primarily owing to endogenous enzymes present in the flour (Loponen et al. 2004; Gänzle et al. 2008). Wheat and rye proteinases exhibit optimum activity at the low pH prevailing during sourdough fermentation. Acidification by lactic fermentation also contributes to the technological properties of doughs made from other cereals, which is increasingly exploited in the emerging market for gluten-free bread (Moroni et al. 2009).
Lactic and acetic acids are primarily responsible for the characteristic taste of sourdough bread. The conversion of glutamine released from cereal proteins to glutamate also contributes to the taste of sourdough bread (Vermeulen et al. 2007). Sourdough fermentation also has a profound influence on the formation of flavor volatiles during dough fermentation and baking. Crumb odor is primarily determined by products of enzymatic and microbial conversions during dough fermentation. Lipid oxidation by cereal enzymes, Ehrlich degradation of amino acids as well as formation of acetyl esters by yeasts, and metabolites from lactic acid bacteria are major contributors to crumb flavor (Hansen and Schieberle 2005). Crust odor is dominated by products of thermal reactions during baking. The proteolytic release of amino acid during sourdough fermentation and particularly the conversion of arginine to ornithine by lactobacilli strongly contribute to flavor generation during baking (Thiele et al. 2002; Hansen and Schieberle 2005).

In addition to the technological benefits and flavor, sourdough bread is characterized by better resistance to microbiological spoilage by molds and rope-forming bacilli (Corsetti et al. 1998; Rosenquist and Hansen 1998). Molds and rope-forming bacilli are primary agents of microbial spoilage of bread. Sourdough technology extends the shelf life without use of added preservatives. These functions of sourdough have become even more important because of the negative

<table>
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<th>Table 12.2</th>
<th>Functions of Sourdough in Bread Making</th>
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<tr>
<td>Leavening action by yeast growing in association with heterofermentative lactic acid bacteria:</td>
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<tr>
<td>Dough easier to bake</td>
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<tr>
<td>Bread crumbs softer and more palatable</td>
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<tr>
<td>Modification of flour components such as swelling and partial hydrolysis of protein and polysaccharides:</td>
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<tr>
<td>Improvement of baking properties of rye dough</td>
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<tr>
<td>Improvement of crumb properties of wheat and rye bread</td>
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<tr>
<td>Control of excessive enzymatic activity of rye flour, especially α-amylase</td>
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<tr>
<td>Starch degradation in wheat breads by using amylolytic strains</td>
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<tr>
<td>Control and inhibition of contaminating or spoiling flora during fermentation, due to organic acids and low pH, and possibly other mechanisms:</td>
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<tr>
<td>Elongation of mold-free time of bread</td>
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<tr>
<td>Prevention of growth of <em>Bacillus subtilis</em>, the rope-causing organism</td>
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<tr>
<td>Accumulation of flavor components such as acetic acid and other fermentation products</td>
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<tr>
<td>Accumulation of flavor precursor compounds such as amino acids and reducing carbohydrates</td>
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<tr>
<td>Increase of mineral bioavailability through degradation of phytate</td>
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<tr>
<td>Characterization of the product by a natural image; greater versatility, local and regional products</td>
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<tr>
<td>Modification of starch structure leading to lower glycemic index values of wheat bread</td>
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</table>
consumer response to additives. The major antimicrobial compound in sourdough is acetic acid. Lactic acid reduces pH and increases the proportion of undissociated acetic acid, which is much more fungistatic than lactate (e.g., Rosenquist and Hansen 1998; Gänzle et al. 1998; Katina et al. 2002). Acidification and acetate levels in sourdough bread suffice to inhibit growth of rope-forming bacilli, but do not fully prevent mold growth (Rosenquist and Hansen 1998; Zhang et al. 2010). Specific antibacterial compounds produced by sourdough lactic acid bacteria include bacteriocins or bacteriocin-like compounds with activity against *Bacillus subtilis* (Corsetti et al. 1996; for a review, see Messens and De Vuyst 2002). Several sourdough isolates of *Lactobacillus reuteri* produce reutericyclin, a heat-stable tetramic acid derivative with broad-range antibacterial activity against gram-positive bacteria (Gänzle et al. 2000). Strain-specific formation of several compounds with antifungal activity by sourdough lactobacilli was also observed (Schnürer and Magnusson 2005). Corsetti et al. (1998) suggested that caproic acid formed by sourdough lactobacilli contributed to antifungal activity. Propionate produced by a mixed culture of *L. buchneri* and *L. diolivorans* delayed fungal growth on rye bread (Zhang et al. 2010). Lavermicocca et al. (2000) identified phenyllactate and hydroxyphenyllactate as new antifungal compounds produced by an *L. plantarum* strain. Although specific starter cultures were shown to effectively delay fungal spoilage of bread, the contribution of most antifungal metabolites to the prevention of fungal spoilage remains unclear (Ryan et al. 2008, 2009).

Sourdough fermentation also improves nutritional properties of bread through phytate degradation and modification of the glycemic index. The extend of phytate degradation and the concomitant increase of the bioavailability of minerals achieved during sourdough fermentation exceeds phytate degradation in straight dough processes owing to increased phytate solubility at low pH and endogenous phytase activity in flour (Hammes and Gänzle 1998; Lopez et al. 2001; Poutanen et al. 2009). Starch bioavailability is reduced by the use of sourdough, and the glycemic response to bread is retarded (Liljeberg and Björck 1994). Several studies confirmed that bread made with sourdough and containing lactic acid produced during fermentation lowers the postprandial glucose and insulin responses in humans (Östman et al. 2002; Poutanen et al. 2009). Altered interaction between starch and gluten due to the presence of lactic acid during baking may account for the reduced glycemic index of sourdough bread (Östman et al. 2002). The selection of starter cultures for fermentative enrichment of sourdough bread with γ-aminobutyric acid or bioactive peptides (Coda et al. 2010) represents an additional strategy to improve the nutritional value of sourdough bread.

Incorporation of suitable dietary oligosaccharides or polysaccharides may provide health benefits by stimulating colonic fermentation to short-chain fatty acids, and by stimulation of beneficial members of intestinal microbiota. This description relates to soluble dietary fiber, resistant starch, and nondigestible oligo- and polysaccharides. Gibson and Roberfroid (1995) suggested the term “colonic foods” or “prebiotics” for food components entering the colon and serving as substrates for the colon microbiota. Prebiotic compounds in cereals are arabinoxylan of rye and wheat, and β-glucan of oat and barley (Jaskari et al. 1998; Crittenden et al. 2002; Karpinnen 2003; Metzler-Zebeli et al. 2010). Exopolysaccharides and oligosaccharides produced by sourdough lactobacilli can additionally provide prebiotic carbohydrates in cereal foods (Schwab et al. 2008; Dlusskaya et al. 2008).

### 12.4.2 Sourdough Fermentation Process

Traditional sourdoughs used in artisanal bread production as well as industrial sourdoughs are maintained by continuous propagation or back-slopping, using the previous batch as an inoculum. These sourdoughs are microbiologically and functionally very stable, demonstrating that the
processes select for microorganisms that are specifically adapted to the substrate and the fermentation conditions (Böcker et al. 1995; Vogel et al. 1999; Meroth et al. 2003; De Vuyst et al. 2009). The periodic analysis of specific sourdoughs that were maintained by continuous propagation over several decades, corresponding to several tens of thousand generations of microbial growth, indicates the stability of the microbiota at the species level or even at the strain level (Spicher and Stephan 1993; Böcker et al. 1995; Gänzle and Vogel 2002). The remarkable stability of sourdoughs that are maintained under carefully controlled conditions also explains why commercial starter cultures consisting of one or more well-defined species or strains of lactic acid bacteria, available as freeze-dried powders or tablets, have not found a substantial market in the baking industry. However, starter cultures containing a stable mixed flora are commercially available. The most successful of these starters is probably the Böcker “Reinzucht” sourdough, which contains Lactobacillus sanfranciscensis (previously Lactobacillus brevis var. lindneri or L. sanfrancisco) as the dominant species (Spicher and Stephan 1993; Böcker et al. 1995; Böcker et al. 1990).

Reflecting the industrial relevance of sourdough fermentations, which was largely limited to rye baking throughout the 20th century, the scientific literature on lactic acid bacteria in sourdoughs has initially been derived from studies on rye sourdoughs produced in central, northern, and eastern Europe. Much of the earlier work was reviewed by Spicher and Stephan (1993) in their book on sourdough technology; the book was recompiled later by Brandt and Gänzle (2005). Reviews on sourdough microbiota of traditional and industrial sourdoughs used have been presented also by Lönner and Ahrné (1995), Gobbetti (1998), Hammes and Gänzle (1998), Vogel et al. (1999), De Vuyst and Vancanneyt (2007) and De Vuyst et al. (2009). Following the growing market for gluten-free cereal foods, recent reviews also focused on the microbiological and technological aspects of gluten-free sourdoughs (Moroni et al. 2009). Information on the microbiology of cereal fermentations in tropical climates is provided in the reviews by FAO (1999), Holzapfel (2002), Blandino et al. (2003), and Nout (2009).

Industrial sourdough fermentation processes vary substantially depending on the scale of the operation, degree of automation, and technological aim of the fermentation. Most sourdough processes are batch processes, although continuous propagation systems for large-scale industrial rye bread production have also been developed and are operating in Europe (Meuser 1995). Sponge dough fermentations, also referred to as type 0 sourdoughs, are widely used in bread baking and in the production of soda crackers (De Vuyst et al. 2009). Sponge doughs are inoculated with baker’s yeast, but prolonged fermentation times (>8 h) invariably also results in the establishment of a population of lactic acid bacteria to cell counts exceeding $10^8$ colony-forming units (CFU)/g. Traditional sourdoughs used as the sole leavening agent, also referred to as type I sourdoughs, are characterized by frequent back-slopping steps at ambient temperatures (Vogel et al. 1999; Häggenman and Salovaara 2008a; De Vuyst et al. 2009). These conditions select for rapidly growing microorganisms. Obligate heterofermentative lactobacilli, particularly L. sanfranciscensis, typically occur in cell counts of $10^8$ CFU/g, whereas sourdough yeasts occur in cell counts ranging from $10^6$ to $10^8$ CFU/g. Industrial sourdoughs for production of baking improvers are typically fermented for elongated fermentation periods to achieve high levels of acidity (Brandt 2007). Industrial sourdoughs, also referred to as type II sourdoughs, are dominated by thermophilic and aciduric obligate heterofermentative lactobacilli. In type II sourdoughs fermented at an elevated temperature ($>35^\circ$C), yeasts are essentially absent (Vogel et al. 1999; Meroth et al. 2003). Gluten-free sourdoughs are produced by technology and fermentation conditions matching type I or type II sourdoughs, but are based on gluten-free cereals maize, rice, sorghum, millet, or teff (Moroni et al. 2009). The use of these cereals in gluten-free sourdough fermentations selects for microbiota that differ from traditional wheat and rye sourdoughs (Vogelmann et al. 2009).
12.4.3 Microecology of Sourdoughs

Whole cereal grains and whole grain wheat or rye flour contains $10^3$–$10^6$ CFU/g of unspecified bacteria and $10^2$–$10^3$ CFU/g of lactic acid bacteria. Spontaneous wheat and rye doughs are fermented by bacilli, Enterobacteriaceae, and lactic acid bacteria, particularly including pediococci, enterococci, and lactococci (Van der Meulen et al. 2007). Back-slopping of the sourdough with a 1–5% inoculum from a previous batch results in dominance of lactic acid bacteria. Obligate and facultative heterofermentative lactobacilli typically account for more than 99.9% of the bacterial microbiota after 5–10 refreshments (Van der Meulen et al. 2007).

Differences in the fermentation processes and the technological aim that were outlined above are reflected by the large diversity of lactic acid bacteria and yeasts isolated from sourdoughs. More than 50 Lactobacillus spp. as well as several Leuconostoc spp., Weisella spp., Pedicoccus spp., and Enterococcus spp. were isolated in high cell counts in sourdough (Vogel et al. 1999; De Vuyst and Neysens 2005; De Vuyst et al. 2009). Recent advances in the molecular taxonomy of lactic acid bacteria resulted in the description of more than 15 new species of Lactobacillus from sourdough in the past two decades (Vogel et al. 1999; De Vuyst et al. 2009). Despite this taxonomic and metabolic diversity of sourdough microbiota, several key organisms were identified that occur frequently in type 0, type I, or type II sourdoughs (Table 12.3).

Sponge doughs are inoculated with high cell counts of baker’s yeast and fermented for 8–24 h, and are particularly used in wheat baking to improve bread flavor (Hansen and Schieberle 2005). The use of sponge doughs in the production of soda crackers also aims to degrade the gluten network (Sugihara 1985). Yeast metabolism creates anaerobic conditions and acidifies the dough to a pH of 5.5, which favor growth of lactic acid bacteria over other facultative anaerobic bacteria that grow in spontaneous sourdoughs. Commercial baker’s yeast preparations are also a likely source of contamination with lactic acid bacteria (Brandt and Hammes 2004). After 24 h of fermentation, cell counts of lactic acid bacteria typically reach $10^9$ CFU/g, and the dough is acidified to pH 4.0–4.5. Facultative heterofermentative lactic acid bacteria, particularly L. plantarum, L. sakei, and pediococci, were frequently isolated from sponge doughs (Table 12.3).

Sourdoughs used as sole leavening agents (type I sourdoughs) typically contain one to three major strains of Lactobacillus spp. and a yeast, Candida humilis or Saccharomyces exiguus. A majority of type I sourdoughs contain L. sanfranciscensis. This particular obligate heterofermentative lactobacillus species was first isolated and described as L. brevis spp. lindneri from German rye sourdoughs, and from the San Francisco French bread process (Sugihara et al. 1970; Kline and Sugihara 1971; Spicher and Stephan 1993). This species was later identified as the key organism in sourdoughs throughout Europe and North America, but has not yet been isolated from any other source. L. plantarum or L. (par)alimentarius are often associated with L. sanfranciscensis in Italian wheat sourdoughs; L. rossiae, L. brevis, or related mesophilic, obligate heterofermentative lactobacilli also occur in type I sourdoughs.

The dominance of L. sanfranciscensis in type I sourdoughs is attributable to its rapid growth in the pH range of 4.0–6.0 at ambient temperatures (Gänzle et al. 1998). L. sanfranciscensis is replaced by thermophilic and acid-tolerant lactobacilli in sourdoughs with extended fermentation times, or at increased fermentation temperatures (Meroth et al. 2003). L. sanfranciscensis has a strong preference for maltose utilization through maltose phosphorylase; some strains lack hexokinase and fail to ferment any other carbohydrate, including glucose (Stolz et al. 1993, 1996; Neubauer et al. 1994; Gobbetti et al. 1995a, 1995b; as reviewed by Gobbetti and Corsetti 1997; Gänzle et al. 2007). L. sanfranciscensis also makes efficient use of electron hydrogen acceptors that are available in wheat and rye doughs to increase the energetic yield in the phosphoketolase
Table 12.3  Typical Species of Lactic Acid Bacteria Detected in Sourdoughs

<table>
<thead>
<tr>
<th>Fermentation Technology</th>
<th>Inoculum and Fermentation Conditions</th>
<th>Key Organisms</th>
<th>Other Frequently Isolated Lactic Acid Bacteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sponge dough fermentation (type 0 sourdough)</td>
<td>Baker’s yeast, single batch fermentation for 8–24 h at ambient temperature</td>
<td>Lactobacillus plantarum</td>
<td>Lactobacillus brevis</td>
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<td></td>
<td></td>
<td>Lactobacillus sakei</td>
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<td></td>
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<td>Pediococcus pentosaceus</td>
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<tr>
<td>Sourdoughs used as sole leavening agent (type I sourdough)</td>
<td>Back-slopping (5–30% inoculum from previous batch of sourdough). Frequent refreshments (2–4 times per day) at ambient temperature corresponding to fermentation times of less than 12 h</td>
<td>Lactobacillus sanfranciscensis</td>
<td>Lactobacillus (par) alimentarius</td>
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<tr>
<td></td>
<td></td>
<td>Lactobacillus brevis</td>
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<tr>
<td></td>
<td></td>
<td>Lactobacillus plantarum</td>
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<td></td>
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<td>Lactobacillus rossiae</td>
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<td></td>
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<td>Weissella confusa</td>
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<td></td>
<td></td>
<td>Leuconostoc mesenteroides</td>
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<tr>
<td>Industrial fermentations for use as baking improver (type II sourdough)</td>
<td>Back-slopping, extended fermentation time to achieve high levels of acidity, often coupled to high fermentation temperatures</td>
<td>Lactobacillus pani</td>
<td>Lactobacillus amylovorus</td>
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<td></td>
<td>Continuous industrial fermentations using high inoculum (50%) and/or high fermentation temperature</td>
<td>Lactobacillus pontis</td>
<td>Lactobacillus acidophilus</td>
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<td></td>
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<td>Lactobacillus delbrueckii</td>
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<td></td>
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<td>Lactobacillus fermentum</td>
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<td></td>
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<td>Lactobacillus reuteri</td>
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pathway. The availability of hydrogen acceptors leads to a shift in heterofermentative metabolism, and acetate rather than ethanol is produced from acetyl phosphate. This shift doubles the yield of ATP in heterofermentative metabolism, but requires an external hydrogen acceptor to regenerate NADH (Kandler 1983; Condon 1987; Axelsson 2004). Oxygen, which is reduced to H₂O by NADH peroxidase, and fructose, which is reduced to mannitol by mannitol dehydrogenase, are major hydrogen acceptors in wheat and rye sourdoughs (Gobbetti and Corsetti 1997; Stolz et al. 1995a, 1995b). Fructose reduction to mannitol accounts for about 50% and 80% of the acetate produced by \textit{L. sanfranciscensis} in wheat and rye sourdoughs, respectively. Other hydrogen acceptors relevant in sourdough include glutathione, α-ketoacids, and aldehydes originating from lipid oxidation (for review, see Gänzle et al. 2007). Understanding the biochemistry of sugar fermentation and acetic acid production in sourdough by heterofermentative lactobacilli has practical uses for improvement of mold-free time, rope prevention, and flavor (see also Table 12.2). In baking applications the addition of fructose in the form of invert sugar has a linear effect on acetate content of sourdough (Röcken et al. 1992).

The yeast present in a sourdough is acid tolerant and typically forms a stable association with lactobacilli. \textit{Candida humilis} (syn. \textit{C. milleri}) and \textit{S. exigua} (syn. \textit{S. minor}, \textit{Torulopsis holmii}, \textit{C. holmii}) are the most typical yeast species in sourdoughs, although \textit{S. cerevisiae} is also detected. There is a nice symbiosis between \textit{C. humilis} and \textit{L. sanfranciscensis} (formerly \textit{L. sanfrancisco}, identical to \textit{L. brevis var. lindneri}). The yeast produces vitamins, and other growth factors required by the lactic acid bacteria (Ng 1972; Gobbetti et al. 1994), whereas the acids produced by the lactic acid bacteria suppress the growth of other microbes. Yeast invertase hydrolyses sucrose as well as the fructo-oligosaccharides such as kestose and nystose that are the major source of fructose in wheat and rye doughs. Hydrolysis of these oligosaccharides supplies fructose for conversion to mannitol by the heterofermentative lactobacilli (Brandt and Hammes 2001). The stable association of yeasts and lactobacilli in type I sourdoughs is also attributable to the lack of competition for nutrients. \textit{L. sanfranciscensis} utilize only maltose while \textit{C. humilis} and other typical sourdough yeasts are incapable of assimilating maltose, thus eliminating competition for the carbon source (Vogel et al. 1999). Likewise, \textit{L. sanfranciscensis} preferentially metabolizes peptides whereas amino acids are the preferred nitrogen source for yeasts (Vermeulen et al. 2005). Sourdough yeasts and \textit{L. sanfranciscensis} have matching growth requirements with respect to temperature, pH, and ionic strength (Gänzle et al. 1998). Experienced bakers adjust the ratio of yeasts to lactobacilli in sourdough, and therewith the level of acidity in the resulting bread, by careful control of temperature, fermentation time, and dough yield (Gänzle et al. 1998).

The microbiota of type II sourdoughs used for the industrial production of baking improvers is not as uniform as the microbiota of type I sourdoughs, reflecting the substantial variation in process conditions that are applied in industrial practice. Many fermentations are characterized by extended fermentation times of 24 h to several days to achieve high levels of acidity. These processes select for acid-tolerant lactobacilli, particularly \textit{L. pontis}, \textit{L. panis}, \textit{L. fermentum}, or \textit{L. reuteri}. The homofermentative organisms \textit{L. amylovorans}, and \textit{L. acidophilus/L. johnsonii} were also frequently isolated from type II sourdoughs (Vogel et al. 1999; Meroth et al. 2003; De Vuyst et al. 2009). The main process parameters differentiating type I and type II sourdoughs are fermentation temperature, and the inoculum level or the fermentation time (Meroth et al. 2003; Vogelmann and Hertel 2011). The metabolism of organisms in the \textit{L. reuteri} group is comparable to \textit{L. sanfranciscensis} with regard to the use of maltose and hydrogen acceptors (Gänzle et al. 2007). However, \textit{L. reuteri} additionally harbors sucrose phosphorylase for efficient sucrose metabolism (Schwab et al. 2007) as well as several mechanisms for acid resistance, which are generally absent in \textit{L. sanfranciscensis}. The conversion of arginine to ornithine, conversion of glutamine to glutamate and
γ-aminobutyrate, as well as the formation of exopolysaccharides all contribute to acid resistance of organisms in the *L. reuteri* group (Rollan et al. 2003; Vermeulen et al. 2007; Stromeck et al. 2011; Kaditzki et al. 2008). The continuous supply of amino acids during prolonged fermentation of wheat and rye sourdoughs by cereal substrates particularly supports the effectiveness of acid resistance mechanisms that are based on conversion of amino acids (Thiele et al. 2002). In some specific processes, a high fermentation temperature is used to achieve accelerated acidification and to control contaminating microbiota (Meuser 1995; Böcker et al. 1995). Fermentation systems operating in the temperature range of 40–50°C suppress the yeast and select for thermophilic organisms such as *L. reuteri*, *L. delbrückii*, or *L. johnsonii* (Meuser 1995; Böcker et al. 1995; Vogel et al. 1999; Meroth et al. 2003).

Gluten-free sourdoughs produced with flour from gluten-free cereals have become available in the past 10 years to cater to the rapidly growing market of gluten-free bread in Europe and North America. The technological function of these sourdoughs, improved texture, flavor, and shelf life of bread, is comparable to the use of sourdough in wheat and rye baking. However, the microbiota of gluten-free sourdoughs are comparable to cereal fermentations in tropical climates that employ the same substrates rather than wheat and rye sourdoughs produced with comparable fermentation conditions (Meroth et al. 2004; Vogelmann et al. 2009; for review, see Moroni et al. 2009; Gänzle and Schwab 2009; Nout 2009). A different carbohydrate supply due to a different level of amylase activities in gluten-free substrates, as well as the presence of antimicrobial polyphenolic compounds in some cereals (e.g., sorghum) may contribute to the selection of fermentation microorganisms that are specific to the cereal substrate (Gänzle and Schwab 2009; Svensson et al. 2010).

12.5 Traditional Lactic Acid–Fermented Cereal-Based Foods Other than Bread

From a global perspective a considerable part of the cereal-based foods made by lactic acid fermentation are products other than sour bread. Such foods include beverages, gruels, dumplings used in stews, and fried products, and some of these foods are staple foods in parts of Africa, Asia, and South America. Maize, sorghum, millet, and other starchy materials are used for the preparation of these indigenous cereal products (Holzapfel 2002; FAO 1999; Blandino et al. 2003; Nout et al. 2007). Fermented beverages and porridges were also known in Europe either as low-alcohol drinks, such as kvass and boza made from wheat or rye (Dlusskaya et al. 2008; Todorov et al. 2008), or sowens, flummeries, and other similar oat-based gruel-type products (Campbell-Platt 1987; Lampinen et al. 1976; Fenton 1974; Salovaara et al. 1991).

Examples of indigenous cereal-based products that have been scientifically studied are ogi and agidi (Nigeria); koko, akassa, and kenkey (Ghana); uji, togwa (East Africa); mawè (Benin); mahewu (southern Africa); idli (India); and mifen (China). Many of these products are also made commercially for local markets (Odunfa and Adyele 1985; Adeyemi et al. 1987; Hounhouigan et al. 1993; Blandino et al. 2003; Nout et al. 2007).

The preparation of fermented sour cereal foods other than bread often follows a simplified pattern: the grains are soaked in clean water for 0.5–3 days. Mixed fermentations, including lactic acid fermentation, take place during the soaking stage. Soaking softens the grains and makes them easier to crush or wet mill into a slurry, from which hulls, bran particles, and germ can be removed by screening and sieving procedures.

Slurrying in water of the material from either wet or dry milling supports fermentation, which is allowed to take place overnight or for longer, usually at ambient temperatures. The slurrying or
doughing stage has many similarities with the sourdough procedures used in traditional European sourdough bread making. Back-slopping, that is, saving part of the previous batch for inoculum for the next batch, may be used. The equipment used may also serve as a source of starter organisms (Wacher et al. 1993; Holzapfel 2002; Hounhouigan et al. 1993). Accumulation of acids occurs much in the same way as in a sourdough for bread. In a fully fermented slurry, the number of lactic acid bacteria may be as many as $10^9$ CFU/g, typical of a fermented sourdough. When the fermentation is completed, the slurry is boiled with an appropriate amount of water so that gelatinization of starch occurs and a product of desired consistency is obtained. The final product may be drinkable, spoonable, or stiff and dumpling-like. The processes and the microorganisms responsible for the souring process have been reported in several studies, including recent reviews by Nout and co-workers (Nout et al. 2007; Nout 2009).

12.6 Cereal-Based Foods Containing Live Lactic Acid Bacteria

Processing of cereal foods is often completed with a final boiling or baking stage to gelatinize starch to give the food the intended texture and to make the food more digestible and palatable. The heat treatment also eliminates the live lactic acid bacteria present in the fermented cereal material. However, there are also processes that involve fermentation after the gelatinizing heat treatment, and hence the product contains live lactic acid bacteria. One example of this type of traditional food is tarhana, a mixture of soured milk and wheat widely consumed in Turkey and elsewhere. The process involves mixing yogurt with wheat flour derived from boiled, dried, and ground wheat grains. The resulting dough is formed into balls and sun-dried to make the tarhana (Certel et al. 2007). Other products of same type are kishk and rabadi (FAO 1999; Elyas et al. 2002).

Lactic acid bacteria are also unavoidably present when local beers are made. Brewing of cereal grains into beer undoubtedly was an integrated alcoholic/lactic fermentation process in its original form. In industrial brewing, lactic acid bacteria are normally considered undesirable contaminants. Lactic acid bacteria are in part responsible for the restricted keeping time of indigenous low-alcohol beers made from various cereals in many parts of the world (Campbell-Platt 1987; Wood 1981; FAO 1999).

Lactic acid fermentation is intentionally utilized in the industrial production of certain cereal-based sour beverage specialties. Representatives of *Lactobacillus* and *Pediococcus* belong to the fermenting flora of lambic beers made from barley and wheat, a specialty of Belgium. Typical of the process is a very long fermentation period, which takes 2 years or more. The result is a fairly strong, sour alcoholic beer sold in bottles. There is a varying live microbial flora in the lambic beers, consisting mainly of various yeasts and lactic acid bacteria (Verachtert and Dawoud 1984; Verachtert and Iserentant 1995).

Sour alcoholic beers made from sorghum and maize are known in Africa. The maize (corn)–based sour nonalcoholic beer, mageu (mahewu), inoculated with a *Lactobacillus* starter, is commercially produced in South Africa (Hesseltine 1979; McMaster et al. 2005). In its original form, mageu was made by adding maize meal to boiling water and cooking for 10 min. Some wheat flour was added to provide amylolytic activity. A spontaneous inoculum was used, *Lactococcus lactis* being the main organism. Industrially produced mageu is made by using starter organisms such as *Lactobacillus plantarum* or *Lactobacillus delbrueckii* (FAO 1999; Hesseltine 1979) and it has also been studied as a vehicle for probiotic *Bifidobacterium* strains (McMaster et al. 2005).

Like fermented milk-based or vegetable-based foods, the lactic-fermented cereal foods also can serve as a vehicle for strains with probiotic properties. An example of such indigenous cereal foods
is togwa, a lactic acid–fermented sorghum or maize gruel, used in Tanzania as a weaning food or as a beverage. Since togwa is not heat treated after fermentation, it contains live lactic acid bacteria such as *L. plantarum* (Mugula et al. 2003).

Kingamkono et al. (1999) found that fermenting togwa inhibited the growth of some enterotoxin-producing bacteria and reported that a significant reduction in the enteropathogen occurrence in rectal swabs of children under 5 years old was achieved when they were fed togwa.

Vogel et al. (1999) pointed out that the predominant strains present in sourdoughs and in other lactic-fermented cereal foods are closely related to or even identical to species found in the animal and human intestinal tract. This is particularly well illustrated using the example of *L. reuteri*, a species that has evolved to colonize the intestinal tract of humans and animals (Walter et al. 2011; Leser et al. 2002) but also occurs in type II sourdoughs as well as in cereal fermentations in tropical climates (see above). *L. plantarum*, a frequent component in sourdoughs and indigenous fermented cereal foods, is also a frequent inhabitant of human intestinal mucosa (Ahrné et al. 1998; Molin 2001). Furthermore, it was shown that an *L. plantarum* strain isolated from a sourdough can become established in human intestinal mucosa after ingestion (Johansson et al. 1993).

Probiotic strains of lactobacilli are generally suitable as starter cultures for cereal-based foods (Molin 2001; Mårtensson et al. 2002; Charalampopoulos et al. 2002a, 2002b; Salovaara and Simonson 2004). An application uses probiotic strains in fermentation of cooked oatmeal and oat bran for the production of yogurt-type alternatives to dairy and soy-based yogurts (Salovaara 1996). It is obvious that many *Lactobacillus* species that are currently used as probiotic adjuncts in dairy foods, particularly *L. reuteri*, *L. casei*, *L. johnsonii*, and *L. rhamnosus* may also serve as starter cultures for probiotic cereal-based foods and beverages (Todorov et al. 2008; Dlusskaya et al. 2008). The cereal grains and their fractions offer a number of substrates for further development of probiotic and other fermented foods (Salovaara and Kurka 1991).

### 12.7 Conclusions

Lactic acid bacteria are utilized in the production of cereal-based products in many ways. Lactic acid fermentation contributes beneficially both to processing technology and to quality of the end products in terms of flavor, keeping properties, safety, and overall image of the product. Among cereal foods, most scientific research and technological development with respect to lactic acid bacteria has been associated with the sourdough bread-making process.

Traditional fermented cereal foods other than bread (i.e., soured porridges and dumplings) have also received some scientific attention. These foods have a major role for millions of people, especially in Africa and Asia, and deserve research and development input to improve the quality and attractiveness of these foods as economical and nutritious staples. One obvious possibility for any market is the use of starters for the fermentation.

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Chapter 13

Lactic Acid Bacteria in Meat Fermentations

Cecilia Fontana, Silvina Fadda, Pier Sandro Cocconcelli, and Graciela Vignolo

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13.1 Introduction

Traditionally, fermentation of meat was considered a method to extend the shelf life of this highly perishable commodity; however, its significance has recently increased as a way of transformation and diversification of meat products. Even when the historical origin of fermented meat products remains unknown, fragmentary bibliographical research traces it back to more than 2500 years in China. Many of these products are known in Europe since the 13th–14th centuries after being introduced by Marco Polo. Proofs of sausage production were first documented in ancient Greece;
this tradition was then inherited by the Romans (Zeuthen 2007). From these times, fermented sausages have spread to central, eastern, and northern European countries, as well as to America and Australia where it is recognized as a European immigrants heritage (Demeyer 2004; Fadda and Vignolo 2007; Vignolo et al. 2010a).

There is renewed interest in traditional, naturally fermented meat products as outlined by the recent abundant literature (Vignolo et al. 2010a,b). The remarkable technological advances and significant improvements in meat hygiene that occurred in the last 50 years have been capitalized for the development of a range of fermented meat products, in which variations in the type and amount of raw materials, fermentation, and drying conditions lead to an extended diversity of products with unique sensorial traits. Nevertheless, the stability of fermented meat products is mainly determined by a combination of acidification brought about by lactic acid bacteria (LAB) and lowering of water activity ($a_w$) during curing and drying. In addition, biochemical and physicochemical changes are produced as a result of the interactions between meat, fat, microorganisms, and processing technology, which as a whole gives rise to the wide range of available fermented sausages. In view of industrially useful innovations, the LAB community existing in fermented meat ecosystems as well as their contribution to the microbiological and physicochemical changes are discussed here.

13.2 Meat Fermentation

Fermentation of meat has been the subject of intensive study over the past decades. Dry and semidry fermented sausages can be defined as a meat product made of a mixture of mainly pork or pork/beef meat, pork fat, salt, curing agents, sugar, spices, to which in many cases starter cultures are added. The mix is then placed into permeable casings and subjected to a variable fermentation and drying and/or smoking process. A well-documented description of sausage manufacture technology can be found elsewhere; thus, only a brief summary is presented.

**Ingredients and additives.** Meat and fat are generally used in a 2:1 ratio; the functional characteristics of meat (composition, pH, and binding properties) are major criteria when selecting meat for fermented sausages production. Although fat content may vary (10–40%), it must have a high melting point and low content of polyunsaturated fatty acids for clear sausage cut. NaCl (2–4%) and nitrite and/or nitrate (150–250 ppm) are also added; microbial growth suppression, $a_w$ reduction, soluble proteins release, and curing red color development are among the main functions during meat fermentation. The use of ascorbates to improve red nitrosylated pigment stability and lipid oxidation prevention, as well as sugars (dextrose, glucose, sucrose/lactose, corn syrup, and starches) to assure rapid acidification has become a common practice. Addition of spices (pepper, paprika, garlic, nutmeg, and clove) is what differentiates fermented sausages and have also proved to act as antioxidants, antimicrobials, and LAB growth stimulators. During the industrial production of fermented sausages, a variable number of other additives, such as artificial colorants, phosphates, flavoring agents, and acidulants are often added (Ruiz 2007; Toldrá 2007; Vignolo et al. 2010a).

The need to standardize processing and quality led to the use of starter cultures, thus avoiding reliance on the “in-house” microbiota or “back-sloping” for fermentation processes. The breakthrough in the use of starter cultures in the United States was achieved as a result of the work of Deibel and Niven (1957), while in Europe the work of Niinivaara (1955) introduced micrococci use to avoid color and flavor defects. After these first experiences, Nurmi (1966) developed mixed cultures composed of lactobacilli and micrococci. Studies on the ecology of fermented
sausages showed that LAB, mainly *Lactobacillus* and coagulase-negative cocci (CNC) represented by *Micrococcaceae*, are the two main bacterial groups technologically important in the fermentation and ripening of sausages (Lebert et al. 2007; Cocconcelli and Fontana 2010).

**Processing technology.** For the manufacture of typical dry and semidry fermented sausages, meat and fat are chilled and comminuted to the desired particle size using a cutter machine. The chopped meat and fat mass is then thoroughly mixed with salt, curing additives, spices, and starter cultures, and quickly after the meat batter is firmly packed in the stuffer to exclude air pockets. Industrially, stuffing by filling natural or synthetic casings is carried out under vacuum. Depending on the type, the sausages are placed in ripening chambers under temperature-, relative humidity (RH)-, and air speed–controlled conditions and subjected to fermentation. Applied conditions vary in terms of temperature and duration; generally, the higher the fermentation temperature the faster the lactic acid production by LAB and color development. For dry sausages this process is usually performed at 12–24°C for 1–7 days, while higher temperatures (25–35°C) and RH between 70% and 95% during a variable processing period are used for semidry sausages. During fermentation, two microbial reactions occur simultaneously and interdependently: the decrease in the pH via glycolysis by LAB and the production of nitric oxide by nitrate- and nitrite-reducing bacteria such as CNC involving *Staphylococcus* and/or *Micrococcus*. Drying of fermented sausages is a key operation in which kinetics and duration vary depending on sausage type. For dry sausages, slow drying rate must be applied (10–15°C for 4–12 weeks) during which a decrease in RH (from 90–95% to 65–75%) and *a*<sub>w</sub> is effected, while fast drying is generally applied to semidry fermented sausages. A critical point in this stage is to avoid the pronounced surface coagulation of proteins so that the water diffusion from the center outward is hindered. When smoke is applied, a contribution to antimicrobial and antioxidant effect, besides generating specific flavor and color components, is achieved. On the basis of the technology used in this stage, two types of products can be distinguished in Europe: Northern and Southern or Mediterranean sausages in which smoking and air-drying, respectively, are applied (Talon et al. 2004; Zukál and Incze 2010; Sikorski and Kolakowski 2010).

### 13.3 LAB Biodiversity in Meat Fermentation

Food ecosystems constitute a large source of microbial diversity in which a number of bacterial communities coexist. In particular, meat ecosystems are able to support the growth of highly specific microbial associations, the presence of which depends on factors that persist during processing, transportation, and storage. Many studies in meat microbiology have established that spoilage is caused by a dominating fraction of the initial microbial association in which high species diversity from a few bacterial genera was reported (Nychas et al. 2007, 2008). Meat spoilage can be considered an ecological phenomenon that encompasses the changes of the available substrates during the prevalence of a particular microbial association, the so-called specific spoilage organisms (Nychas et al. 2008). The ecological strategies adopted by microorganisms to grow in meat ecosystems are the consequence of the prevailing environmental conditions, in which the intrinsic and extrinsic factors governing microbial growth will determine the type and number of bacteria present in meat. Among these factors, physicochemical ones (concentration and availability of nutrients, pH, redox potential, buffering capacity, *a*<sub>w</sub>, meat structure) and those related to storage and processing conditions (temperature and oxygen availability) are the most prevalent. The selective influence of the previously outlined factors on meat as a substrate may determine the different meat ecosystems (chilled packaged raw meat, cooked meat, and fermented meat), but
they do not explain why meat and meat products specifically select microorganisms not isolated from other food products.

The presence of ecological determinants influences the establishment of a specific microbial consortium that will determine the rate of colonization. The microbiota that participates in the fermentation and ripening of sausages is highly dependent on the technology used. Sodium chloride, nitrate/nitrite, sugars, and the particular $a_w$ (0.85–0.92), temperature (12–18°C to 24–30°C), and oxygen gradients during ripening will highly select a microbiota able to develop in this ecological niche. In traditional fermented sausages, *Lactobacillus sakei*, *Lactobacillus curvatus*, *Lactobacillus plantarum*, and *Pediococcus* spp. are by far the most often isolated species among LAB, which must have adapted to the existing stringent conditions during meat fermentation. As a versatile bacterium, *L. plantarum* has been identified as part of the fermented sausages microbiota; however, this species lacks the meat specialization found in *L. sakei* (Chaillou et al. 2005, 2009; Hufner et al. 2007). Less information is available on the physiology and genetics of *L. curvatus* other than the production of antibacterial substances. Although pediococci do not compose a relevant part of the microbial community of European fermented sausages, they are used as a starter culture in North American sausage style. Moreover, during spontaneous meat fermentation, enterococci, particularly *Enterococcus faecium* represent another LAB species that can be found in relatively high numbers, contributing together with lactobacilli to meat fermentation (Hugas et al. 2003).

In the last decades, microbial ecology has undergone profound changes as the result of the huge impact of emerging technologies applied to the analysis of natural communities. New molecular and genomic approaches are revealing a more dynamic and diverse view of the microbial world than originally anticipated. Culture-dependent and -independent molecular methods have provided a better knowledge of the microbial biodiversity in a wide range of environments, establishing a link between function and complex relationships among the members of natural microbial consortia (Vignolo et al. 2010a). Moreover, the genome sequencing of *L. plantarum* (Kleerebezem et al. 2003), *L. sakei* (Chaillou et al. 2005), as well as *Pediococcus pentosaceus* (Makarova et al. 2006) represented a great help in the design of appropriate primers and probes for the molecular identification, control, and monitor of LAB species in fermented sausage ecosystem. The information existing on this ecosystem was obtained by using a combination of traditional and molecular culture-dependent and -independent methods and is shown in Table 13.1. From the picture of fermented sausage microbiota, the predominance of *L. sakei* and *L. curvatus* emerges. The isolation frequency of *L. plantarum* was not as high as that of the other two lactobacilli, although it has also been identified as an important species participating in sausage fermentation. In significantly lower numbers, other *Lactobacillus* species were also identified, such as *Lactobacillus bavaricus* (Hugas et al. 1993; Coppola et al. 2000; Kozackinski et al. 2008); *Lactobacillus casei* and *Lactobacillus paracasei* (Cocolin et al. 2000, 2004; Andrighetto et al. 2001; Papamanoli et al. 2003; Drosinos et al. 2005; Rantsiou et al. 2005a,b; Kozackinski et al. 2008); *Lactobacillus brevis* (Cocolin et al. 2000; Drosinos et al. 2005; Comi et al. 2005; Benomo et al. 2008; Kozackinski et al. 2008); *Lactobacillus paraplantarum* (Comi et al. 2005; Rantsiou et al. 2005a); *Lactobacillus alimentarius* (Cocolin et al. 2000; Kozackinski et al. 2008); *Lactobacillus rhamnosus* (Drosinos et al. 2007; Kozackinski et al. 2008); *Lactobacillus buchneri* (Papamanoli et al. 2003); *Lactobacillus pentosus* and *Lactobacillus fermentum* (Drosinos et al. 2005; Kozackinski et al. 2008). In regard to pediococci, *P. pentosaceus* and *Pediococcus acidilactici* were often isolated during fermentation of European sausages (Benito et al. 2007; Bonomo et al. 2008; Kozackinski et al. 2008; Albano et al. 2008).

On the other hand, significant differences in the presence and persistence of enterococci were reported in fermented sausages (Samelis et al. 1998; Papamanoli et al. 2003; Aymerich et al. 2003;
Table 13.1 LAB Species Isolated from Traditional Fermented Sausages

<table>
<thead>
<tr>
<th>Source</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>L. sakei/curvatus/plantarum/bavaricus</td>
<td>Hugas et al. (1993)</td>
</tr>
<tr>
<td>L. sakei/curvatus/plantarum; Carnobacterium sp.; Enterococcus sp.</td>
<td>Samelis et al. (1998)</td>
</tr>
<tr>
<td>L. sakei/curvatus; Pediococcus sp.</td>
<td>Santos et al. (1998)</td>
</tr>
<tr>
<td>L. sakei/curvatus/bavaricus;</td>
<td>Coppola et al. (2000)</td>
</tr>
<tr>
<td>L. sakei/curvatus/plantarum/casei/brevis alimentarius</td>
<td>Cocolin et al. (2000)</td>
</tr>
<tr>
<td>L. sakei/plantarum/curvatus; Leuc. carnosum/gelidum/pseudomesenteroides; P. pentosaceus</td>
<td>Parente et al. (2001)</td>
</tr>
<tr>
<td>L. sakei/curvatus/plantarum/paracasei</td>
<td>Andrichetto et al. (2001)</td>
</tr>
<tr>
<td>L. sakei/curvatus/plantarum</td>
<td>Cocolin et al. (2001)</td>
</tr>
<tr>
<td>L. sakei/curvatus/plantarum/buchneri/paracasei; E. faecium; Pediococcus sp.; Leuconostoc sp.</td>
<td>Papamanoli et al. (2003)</td>
</tr>
<tr>
<td>L. sakei/curvatus/plantarum; E. faecium</td>
<td>Aymerich et al. (2003)</td>
</tr>
<tr>
<td>L. sakei/curvatus/casei; E. casseliflavus; Leuc. mesenteroides; Lac. lactis</td>
<td>Cocolin et al. (2004b)</td>
</tr>
<tr>
<td>L. sakei/curvatus/plantarum/brevis/paraplantarum; Lac. lactis; E. pseudoavium; Leuc. citreum/mesenteroides; W. hellenica</td>
<td>Comi et al. (2005)</td>
</tr>
<tr>
<td>L. sakei/curvatus/plantarum/casei/paracasei; Lac. lactis; Leuc. citreum/pseudoavium; Weissella sp.</td>
<td>Rantsiou et al. (2005a)</td>
</tr>
<tr>
<td>L. sakei/curvatus/plantarum; Lac. garviae</td>
<td>Rantsiou et al. (2005b)</td>
</tr>
<tr>
<td>L. sakei/curvatus/plantarum; Enterococcus</td>
<td>Fontana et al. (2005)</td>
</tr>
<tr>
<td>L. sakei/plantarum/paracasei/pentosus/brevis/rhamnosus; Lac. lactis; E. faecium; Leuconostoc sp.</td>
<td>Drosinos et al. (2005, 2007)</td>
</tr>
<tr>
<td>L. sakei/curvatus; Leuc. mesenteroides</td>
<td>Aymerich et al. (2006)</td>
</tr>
</tbody>
</table>

(continued)
Cocolin et al. 2004; Comi et al. 2005; Fontana et al. 2005; Rantsiou et al. 2005; Kozačinski et al. 2008; Albano et al. 2008). In spite of the controversial presence of Enterococcus in foods, E. faecium from meat was shown to have a lower pathogenic potential than clinical strains, and indeed some of them are used as starter cultures owing to their competitiveness in the meat environment and their contribution to final flavor and safety (Hugas et al. 2003; Franz et al. 2010). As shown in Table 13.1, species from the genera Leuconostoc, Carnobacterium, and Weissella were sporadically isolated, underlining possible pitfalls in their identification or simply their low incidence in this ecological niche; these LAB genera are being considered as undesirable spoilage during meat fermentation. Since the main species found in the sausages studied thus far were the same among the different products, it can be suggested that production plant–specific biotypes develop within the species (Rantsiou et al. 2005). This microbiota, usually referred to as the “house flora,” and resulting from the selective action of processing conditions, will predominate during fermentation and characterize the final product of a specific plant. These well-adapted biotypes are promising strains for the development of autochthonous starter cultures, which will enable sausages to be produced with both high hygienic and sensory quality (Casaburi et al. 2007; Talon et al. 2008).

13.4 Functionality of LAB in Meat Fermentation

In recent years, much attention has been paid to functional properties of LAB and efforts have been made by the food industry to develop new products with increased health benefits by introducing LAB strains having technological advantages. Nowadays, it is accepted that appropriate
starter cultures have to be selected from the indigenous populations present in food products in order to be more competitive, well-adapted, and with high metabolic capacities to beneficially affect quality and safety while preserving typical properties of a particular food (Leroy et al. 2006; Talon et al. 2008). Indeed, research on the technological potential of LAB and their adaptive mechanisms to a particular environment has been of utmost interest for the starter cultures industry (Aymerich et al. 2003). The most significant functional properties of LAB during meat fermentation as main criteria for the selection of starter cultures are discussed here.

13.4.1 LAB Competitiveness in the Meat Environment

During meat fermentation, competitiveness is strictly related to the ability of LAB to adapt to the existing environmental conditions during this process. Variations in temperature, osmotic conditions, and oxidative or acidic environments are situations to which LAB are routinely subjected during meat processing. Different approaches were used to understand adaptation of bacteria to stressful environments. Currently, the proteomic approach represents a valuable tool to understand adaptive mechanisms contributing to establish the connection between genome sequences and their biological role (Champomier-Vergès et al. 2010).

*L. sakei*, recognized as the LAB strain most often used as starter culture for fermented sausage production, has evolved an adaptation to the meat environment, harboring the genetic function that gives it the ability to grow and survive in this ecological niche. In particular, the environmental conditions during sausage fermentation were shown to induce the expression of 15 genes in *L. sakei* (Hüfner et al. 2007). Consistent with the expected metabolic adaptation, these genes code for proteins involved in amino acids and carbohydrate transport, lipid metabolism, and stress response; the inactivation of the heat shock regulator gene *ctsR* resulted in an improved growth of *L. sakei* in fermented sausages. Proteomic studies have been carried out to evaluate *L. sakei* adaptation to the stressful conditions such as cold temperature, high NaCl concentration, oxidative shock, and high hydrostatic pressure (Champomier-Vergès et al. 2002; Marceau et al. 2004; Jofré et al. 2007). Its psychrotrophic character and salt tolerance may be due to its ability to efficiently accumulate osmo- and cryo-protective solutes such as betaine and carnitine, and to its cold stress response; *L. sakei* has more putative cold stress genes than any other lactobacilli (Chaillou et al. 2005). A combination of mechanisms, including modification of carbohydrate metabolism (down-regulation of glycolysis) and stimulation of oxidative stress may also increase its resilience to cold. A proteomic approach to understand *L. sakei* 23K growth response in presence of meat proteins was used by Fadda et al. (2010a) in which the activity of muscle endogenous enzymes in cooperation with the up-regulated peptidases from *L. sakei* was demonstrated. Supplementary nutrients furnished by meat extracts were responsible for the overexpression of proteins involved in translation, peptide/amino acid metabolism, and energy production. Conversely, under-regulation of stress proteins suggested that sarcoplasmic and myofibrillar extracts would not represent a stress environment per se for *L. sakei*, constituting a regulated state not subjected to a stress-induced response, in contrast to the harsh conditions during fermentation. In addition, the relatively abundant arginine concentration in muscle myofibrillar proteins was reported to be used by *L. sakei* as an energy source in the absence of glucose (Champomier-Vergès et al. 1999). In fact, *L. sakei* genome analysis has shown the presence of genes coding for enzymes involved in arginine metabolism, which increases the competitiveness of this bacterium in the meat environment (Chaillou et al. 2005). Technological conditions constitute the main inducers for bacterial adaptive responses reported thus far. The transference of genomic information into useful biological insight is an
important step to achieve better product quality through the selection of well-adapted strains and optimizing the production process.

13.4.2 Acid Production

During fermentation and ripening, LAB convert glucose (the primary energy source) to lactic acid, which is mainly responsible for the pH decrease. As glucose is rapidly consumed in meat, the presence in *L. sakei* of catabolic genes coding for adenosine deaminase, inosine hydrolase, and nucleoside phosphorylase was reported to enable the release of ribose for energy production (Chaillou et al. 2005). This is an example of *L. sakei* adaptation to the meat environment, improving acidification as a result of an alternative pathway. In addition, the presence of methylglyoxal synthase has been proposed to counteract frequent glucose starvation and modulate metabolism of alternative carbon sources (Chaillou et al. 2005). A proteomics approach was used by McLeod et al. (2010) to compare 10 *L. sakei* food isolates regarding their metabolic routes when growing on glucose and ribose. Ten proteins were shown to be up-regulated in most of the strains after growth on ribose; three of them were directly involved in ribose catabolism. Moreover, it was observed that this bacterium altered its pyruvate metabolism when growing on ribose compared with glucose, possibly due to an increased ATP generation from pyruvate per ribose unit (Axelsson 2004). On the other hand, the improvement of *L. sakei* acidification performances during fermentation was reported after environmental challenges such as cold and osmotic shocks (Hüfner and Hertel 2008).

13.4.3 Catalase and Superoxide Dismutase Activities

The metabolism of most LAB, such as the adventitious lactobacilli that contaminate raw meat, may lead to the formation of hydrogen peroxide (H$_2$O$_2$), a compound that interferes with the sensorial properties of meat products, being involved in discoloration of nitroso heme pigment and lipid oxidation. Catalase plays an important role in reducing oxidative stress by decomposing H$_2$O$_2$ to water and oxygen; thus its production is considered a relevant technological property of starter cultures for fermented meat products (Leroy et al. 2006). The presence of a heme-dependent catalase has been demonstrated in *L. plantarum* (Igarashi et al. 1996) and *L. sakei* (Noonpakdeeaa et al. 1996), the catalase is active in meat products because of the abundant presence of heme sources (Hertel et al. 1998). Moreover, analysis of the *L. sakei* genome revealed that this meatborne organism harbors systems for the protection against reactive oxygen species, such as Mn-dependent superoxide dismutase, heme-dependent catalase, and NADH oxidase (Chaillou et al. 2005). Recently, genes encoding catalase have been successfully expressed in heterologous hosts to improve the oxidative stress resistance of LAB strains (An et al. 2010).

13.4.4 Meat Protein Degradation by LAB: Role in Flavor Development

Proteolysis of meat proteins has been widely studied, and the contribution to flavor of many of these proteolytic fractions such as peptides and amino acids is generally accepted (Sentandreu et al. 2007). The ability of some lactobacilli strains to hydrolyze pork muscle sarcoplasmic proteins releasing peptides that could play a role in the taste has been reported by Fadda et al. (2010b). Indeed, the intracellular amino, di-, and tripeptidases of *Lactobacillus* were reported as being responsible for the generation of small peptides and amino acids, which contribute either as direct
flavor enhancers or as precursors of other flavor compounds during the ripening of fermented sausages. No extracellular proteases have been characterized thus far from meatborne lactobacilli; in fact, the genome of *L. sakei* 23K has not revealed genes coding for any extracellular protease (Chaillou et al. 2005). Different studies showed that initial myofibrillar protein hydrolysis was not significantly affected by LAB; the tissue proteolytic system is responsible for such activity (di Cagno et al. 2008; Fadda et al. 2010b; Spaziani et al. 2009). However, a major role in secondary proteolysis was attributed to microbial peptidase activities; free amino acids were released from muscle proteins due to hydrolysis becoming dominant during late ripening, thus contributing to the aged flavor of fermented sausages (Casaburi et al. 2008; di Cagno et al. 2008; Visessanguan et al. 2004). Moreover, changes in free amino acids in fermented sausages due to the action of different starter formulations (including *L. curvatus* and *Staphylococcus xylosus*) were reported by Casaburi et al. (2008). Nevertheless, sarcoplasmic protein hydrolysis was evident in samples containing lactobacilli, which indicates that proteolysis was carried out by both microbial activity and endogenous proteases activated by pH decrease. In fact, acid production due to the acidogenic metabolism of LAB during sausage fermentation cannot be excluded from the global proteolytic event, which may overlap bacterial hydrolytic activity (Fadda et al. 2010c).

### 13.4.5 Bacteriocin Production and Biopreservation

On the basis of the wide spectrum of produced antimicrobial compounds, LAB can be exploited as microbial cell factories for food biocontrol (Gálvez et al. 2010). The antibacterial activity of organic acids produced by LAB and the ability to decrease pH during fermentation constitute the main mechanism for biopreservation of foods. However, specific strains of LAB are further known to produce other antimicrobial substances such as low molecular weight metabolites, hydrogen peroxide, antifungal compounds, bacteriocins, and bacteriocin-like molecules, which can be exploited for effective competitive exclusion. Bacteriocins produced by LAB are a heterogeneous group of peptides, and because new bacteriocins are continuously being discovered their classification has been changing. Cotter et al. (2005) suggested some modifications to Klaenhammer’s classification scheme, resulting in only two principal categories: the lanthionine-containing bacteriocins (Class I), and the non-lanthionine-containing bacteriocins (Class II) involving IIa, IIb, IIC, and IIId subclasses and the bacteriolysins group. Later on, Heng et al. (2007) retained Class III for the division into IIIa (bacteriolysins) and IIIb (nonlytic proteins), adding Class IV for cyclic bacteriocins. Moreover Nissen-Meyer et al. (2009) suggested a division of Class II bacteriocins into four subgroups according to similarities of their C-terminal regions, while a subdivision of the bacteriocins from gram-positive bacteria into 12 groups supported by structure-based sequence fingerprints was recently reported.

Fermented sausages are known to be shelf-stable products, in which a sequence of inhibitory hurdles (NaCl, NO$_2$/NO$_3$, low Eh and $a_w$) is present. *Staphylococcus aureus*, *Salmonellae*, and *Clostridium perfringens*, all known to cause food poisoning, have been traditionally implicated in fermented sausage contamination, while *Listeria monocytogenes* and shiga toxin–producing *Escherichia coli* have recently emerged as major public health concerns. However, hurdles during the manufacturing process may not be sufficient to prevent the survival of pathogens; an additional hurdle to reduce contamination risk would be the use of competitive bacteriocin-producing starter cultures referred to as bioprotective cultures. The use of bacteriocinogenic LAB strains as functional starter culture or coculture for sausage fermentation must be able to optimally drive the fermentation process, producing acid beyond the ability to produce enough bacteriocins in order to afford bioprotection (Vignolo et al. 2008). Bacteriocin-producing LAB associated with fermented
### Table 13.2 Examples of Bacteriocins Produced by LAB Isolated from Fermented Meat Products

<table>
<thead>
<tr>
<th>Bacteriocin</th>
<th>Class</th>
<th>Produced By</th>
<th>Isolated From</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nisin</td>
<td>Ia</td>
<td>Lac. lactis BB24</td>
<td>Spanish ferm sausages</td>
</tr>
<tr>
<td>Nisin Z</td>
<td>Ia</td>
<td>Lac. lactis WNC20/TFF221</td>
<td>Thai ferm sausages</td>
</tr>
<tr>
<td>Lactocin S</td>
<td>Ia</td>
<td>L. sakei 148, V18</td>
<td>Spanish ferm sausages</td>
</tr>
<tr>
<td>Lactocin S</td>
<td>Ia</td>
<td>L. sakei L45</td>
<td>Norwegian ferm sausages</td>
</tr>
<tr>
<td>Lactocin 705</td>
<td>IIb</td>
<td>L. curvatus CRL705</td>
<td>Argentinean ferm sausages</td>
</tr>
<tr>
<td>Sakacin P</td>
<td>IIa</td>
<td>L. sakei I151</td>
<td>Italian ferm sausages</td>
</tr>
<tr>
<td>Sakacin A</td>
<td>IIa</td>
<td>L. sakei Lb706</td>
<td>Meat products, beef,</td>
</tr>
<tr>
<td>Sakacin K</td>
<td>IIa</td>
<td>L. sakei CTC494</td>
<td>Spanish ferm sausages</td>
</tr>
<tr>
<td>Brevicin 27</td>
<td>IIId</td>
<td>L. brevis SB27</td>
<td>Ferm sausages</td>
</tr>
<tr>
<td>Curvacin A</td>
<td>IIa</td>
<td>L. curvatus LTH1174</td>
<td>German meat products</td>
</tr>
<tr>
<td>Curvaticin L442</td>
<td>IIa</td>
<td>L. curvatus L442</td>
<td>Greek ferm sausages</td>
</tr>
<tr>
<td>Plantaricin A</td>
<td>IIId</td>
<td>L. plantarum CTC305</td>
<td>Spanish ferm sausages</td>
</tr>
<tr>
<td>Plantaricin UG1</td>
<td></td>
<td>L. plantarum UG1</td>
<td>Dry-ferm sausage</td>
</tr>
<tr>
<td>Plantaricin Lp31</td>
<td></td>
<td>L. plantarum Lp31</td>
<td>Argentinean ferm sausages</td>
</tr>
<tr>
<td>BacST202/216Ch</td>
<td></td>
<td>L. plantarum ST202/216Ch</td>
<td>Portugal Chouriço/ Beloura</td>
</tr>
<tr>
<td>Pentocin 31-1</td>
<td></td>
<td>L. pentosus 31-1</td>
<td>Chinese ferm meat product</td>
</tr>
<tr>
<td>Pentocin 31-1</td>
<td></td>
<td>Leuc. mesenteroides L124/E131</td>
<td>Greek ferm sausages</td>
</tr>
<tr>
<td>Pediocin PA-1</td>
<td>IIa</td>
<td>P. acidilactici HA-6111-2/ HA-5692-3</td>
<td>Portugal Alheira ferm sausages</td>
</tr>
<tr>
<td>Pediocin PA-1</td>
<td>IIa</td>
<td>P. acidilactici PAC1.0</td>
<td>American-style sausages</td>
</tr>
<tr>
<td>Pediocin PA-1</td>
<td>IIa</td>
<td>P. pentosaceous Z102</td>
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<tr>
<td>Pediocin L50</td>
<td>IIc</td>
<td>P. acidilactici L50</td>
<td>Spanish ferm sausages</td>
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<tr>
<td>Enterocin L50/P</td>
<td>IIb/c</td>
<td>E. faecium L50</td>
<td>Spanish ferm sausages</td>
</tr>
<tr>
<td>Enterocin A/B</td>
<td>IIa/c</td>
<td>E. faecium CTC492</td>
<td>Spanish ferm sausages</td>
</tr>
</tbody>
</table>

(continued)
Lactic Acid Bacteria in Meat Fermentations

SAUSAGES ARE MAINLY FROM CLASS I AND II; EXAMPLES OF THEM ARE SHOWN IN TABLE 13.2. LAB CURRENTLY ASSOCIATED WITH MEAT FERMENTED PRODUCTS, *L. sakei*, *L. curvatus*, *L. plantarum*, *P. acidilactici*, AND *E. faecium*, SHOWED A GREAT POTENTIAL FOR BACTERIOCIN PRODUCTION, THIS BEING A COMMON PHENOTYPE. BECAUSE BACTERIOCINOGENIC LAB ARE MORE COMPETITIVE THAN NON-BACTERIOCIN-PRODUCING VARIANTS, THE APPLICATION OF SUCH STRAINS AS A FUNCTIONAL STARTER CULTURE MAY IMPROVE THEIR COMPETITIVENESS, LEADING TO A MORE CONTROLLED AND STANDARDIZED FERMENTATION PROCESS.

FOR A MORE IN-DEPTH DISCUSSION ON LAB BACTERIOCINS, SEE THE CHAPTER ON THIS TOPIC ELSEWHERE IN THIS BOOK.

### 13.4.6 Probiotics LAB in Meat Products: Reality or Fantasy?

Probiotic organisms are defined as “nonpathogenic microorganisms that, when ingested in certain numbers exert a positive influence on host physiology and health beyond inherent general nutrition” (Ouwehand et al. 2002). The use of fermented sausages as probiotic food carriers has been postulated since these products are not heated and harbor high numbers of LAB. However, to use probiotics in fermented sausages, several characteristics of the culture, in addition to technological, sensory, and safety properties, should be taken into account. The survival of probiotic lactobacilli through the gastrointestinal tract was reported to be protected by the sausage matrix (Klingberg and Budde 2006). Although probiotic viability may be reduced due to the high curing salt content and the low \( a_w \) and pH, human intestinal lactobacilli have been shown to survive the sausage manufacturing process and can be detected in high numbers in the final product (De Vuyst et al. 2008). LAB strains isolated from sausages have been characterized as probiotic (Papamanoli et al. 2003; Pennacchia et al. 2006; Rebucci et al. 2007); the evaluation of LAB strains to be used in Iberian dry-fermented sausages showed that only the preselected meatborne *P. acidilactici* SP979 was able to perform during fermentation without physicochemical and sensory modifications (Ruiz-Moyano et al. 2011). Similarly, when the potential of *Pediococcus* strains isolated from Turkish-type fermented sausages (sucuk) were evaluated as probiotics, they were able to survive at low pH values and in the presence of bile salts, and were able to autoaggregate and coaggregate with *Lis. monocytogenes* (Yuksekdog and Aslim 2010). Moreover, an approach combining proteomic and phenotypic analyses of the meatborne *L. sakei* 23K allowed defining functions important for improving its fitness during gastrointestinal tract colonization (Chiaramonte et al. 2010). The vast quantity of artisan-fermented sausages from different origins represents a biodiversity treasure that can be exploited to create functional starter cultures with high impact on safety and health.
13.5 Safety of LAB in Fermented Meat Products

Knowledge of safety and technological properties of indigenous microbiota from fermented meat is of practical relevance for improvement of starter culture technology and production of fermented meat products. Although LAB have a long history of safe use and association with human food production and health, the biogenic amines (BA) content as well as the presence of transmissible determinants for antibiotic resistance in fermented meat products are among the risk factors of major concern. The safety of *Lactobacillus* and *Pediococcus* species, as the main LAB involved in meat fermentation, was recently evaluated by a risk assessment approach to have Qualified Presumption of Safety (European Food Safety Authority [EFSA] 2010). However, the presence of enterococci in food is highly controversial due to some strains harboring virulence determinants, being resistant to a wide variety of antibiotics, and being opportunistic human pathogens and cause of nosocomial infections.

For a more in depth discussion on the safety of LAB, see the chapter on this topic elsewhere in this book.

13.5.1 BA Production

During meat fermentation, microbial growth, acidification, and proteolysis provide favorable conditions for BA production. The large quantities of proteins present and the proteolytic activity found during ripening of meat products provide the precursors for decarboxylase reactions performed by members of the meat microbiota (Suzzi and Gardini 2003; Komprda et al. 2004). The presence in food of BA, such as cadaverine, putrescine, spermidine, histamine, phenethylamine, agmatine, and tyramine, is a potential health concern because their consumption can be toxic (Suzzi and Gardini 2003). The final BA content in fermented sausages depends on the microbial composition of raw meat, the fermentation conditions, and the starter culture inoculated. Recently, Latorre-Moratalla et al. (2010) reported high aminogenic activity, in particular for the production of tyramine, β-phenylethylamine, tryptamine, and the diamines putrescine and cadaverine, among LAB isolated from European fermented sausages. *L. curvatus* and enterococci were found to be the most aminogenic species and genus, respectively, while *L. sakei* and *L. plantarum* would be the most suitable for use as starter cultures as far as low BA production is concerned. Enterococci strains isolated from fermented sausages are well known for their capacity to produce BA (Bover-Cid et al. 2001; Hugas et al. 2003). The use of decarboxylase-negative starter cultures that are highly competitive and fast acidifiers were reported to prevent the growth of BA producers, leading to end products nearly free of them, as long as high-quality raw materials and appropriate technological conditions are used (González-Fernández et al. 2003; Leroy et al. 2006; Coloretti et al. 2008). Another alternative to decrease BA production during meat fermentation might be the introduction of starter strains possessing amine oxidase activity (Fadda et al. 2001; Suzzi and Gardini 2003).

13.5.2 Antibiotic Resistance

The emergence and spread of antimicrobial-resistant bacteria constitute an increasing public health concern. Clinical and veterinary use of antimicrobials for therapeutic or prophylactic purposes as well as for growth promotion in animal husbandry contributes to this trend. LAB may act as reservoirs of antimicrobial resistance genes that might be transferred to commensally or pathogenic bacteria (Franz et al. 2010). Antibiotic resistance in LAB from meats and meat products such as *L. sakei*, *L. curvatus*, and *L. plantarum* has been reported (Gevers et al. 2003a,b; Zonenschain et
al. 2009). The contribution of food enterococci to the occurrence and spread of antibiotic resistance and virulence was further examined, in particular meat enterococcal strains that are mostly resistant to clinically relevant antibiotics (Hugas et al. 2003; Franz et al. 2010). *Pediococcus* species have been described as intrinsically resistant to streptomycin, kanamycin, tetracycline (especially *P. acidilactici*), vancomycin, and ciprofloxacin (Scientific Committee on Animal Nutrition 2005). Danielsen et al. (2006) examined the susceptibility of six *Pediococcus* spp. to 14 antimicrobial agents, in which 30 out of 31 strains appeared to be safe to use because of the absence of acquired resistance genes. Recently Cordeiro et al. (2010) reported the susceptibility of meat starter cultures to antimicrobials used in food animals in Canada, the resistance incidence being higher among *P. pentosaceus* and lower for *Staphylococcus carnosus* strains. Among the LAB strains involved in meat fermentation, genetic determinants for chloramphenicol acetyltransferase (*cat*-TC), erythromycin (*erm* B), and tetracycline (*tet* L, *tet* M, *tet* S) resistance have been identified, suggesting that horizontal gene transfer may have occurred (Gevers et al. 2003b; Ammor et al. 2008; Zonenschain et al. 2009). It was reported that, even in the absence of selective pressure, mobile genetic elements carrying antibiotic resistance can be transferred at a high frequency among the microbial community during sausage fermentation (Vogel et al. 1992; Cocconcelli et al. 2003). Indeed the food chain has been recognized as one of the main routes for the transmission of antibiotic-resistant bacteria between animal and human populations (Witte et al. 2000). EFSA (2010) has recently concluded that bacteria deliberately introduced in the food chains, such as starter cultures, might pose a risk to human and animal health because they carry acquired resistance genes.

**References**


Lactic Acid Bacteria: Microbiological and Functional Aspects


Lactic Acid Bacteria in Meat Fermentations


Chapter 14

Examples of Lactic-Fermented Foods of the African Continent

Charles M.A.P. Franz and Wilhelm H. Holzapfel

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</table>

14.1 Introduction

Our well-being is influenced by various microbial activities, which are perhaps best appreciated in relation to beneficial changes observed during food fermentations since the earliest human history. The positive perceptions of microbes in our time are to a large extent derived from advantages offered by food fermentations well known and practiced in practically all human cultures and traditions. Beneficial effects are exerted particularly by microorganisms such as lactic acid bacteria (LAB) and some yeasts. Traditional fermentation processes such as sauerkraut, kimchi, and dairy
fermentations in industrialized countries, and numerous other processes in developing countries, offer interesting and valuable study models for understanding the underlying mechanisms of protection and enrichment of our daily diet. Understanding these mechanisms provides a valuable scientific basis for biological approaches toward safeguarding of foods, and for the protection of the human host through interactions in the gastrointestinal tract (GIT). Food fermentations are diverse, ranging from LAB-dominated fermentations in the African continent to Asian fermentations mainly associated with molds and yeasts. Moreover, LAB are associated with different traditions of production across different regions. In these fermentations, a wide range of raw materials and processes are being used.

The fermentation of food raw materials offers numerous benefits, several of which are vital for survival and safe nutrition of populations in traditional communities. Here they are treasured as major dietary constituents, primarily because of their stability and elevated shelf life under ambient conditions, and for their safety and traditional acceptability. In addition to these basic advantages, even modern societies in industrialized countries appreciate the enrichment of the diet and the widely proclaimed health attributes of lactic-fermented foods. Examples of beneficial aspects (Steinkraus 1995; Holzapfel 1997; Leroy and De Vuyst 2004), some of which are not always realized by industrialized societies, include

- Preservation/safeguarding by (a) competition and (b) via metabolic activities by metabolic products such as lactic acid, acetic acid, CO₂, bacteriocins, and low molecular weight metabolites
- Improvement/enrichment of the diet (quality—sensory, structural)
- Improvement of digestibility → reduction of preparation time and of the energy required
- Biological enrichment (protein; essential amino acids, essential fatty acids, and vitamins)
- Detoxification and/or degradation of antinutritive factors (of major importance in developing societies)
- Functional (probiotic) effects
- Decrease in cooking time and fuel requirements

The most important benefits of fermented foods are in several ways related to the GIT and our well-being, and are associated with lactic fermentations. Reduction of the pH to a “safe” level of 4.5 or lower (Holzapfel 1997) is a result either of homo- or heterofermentation, or both. In addition, the competitive effect of well-adapted LAB will eliminate or inhibit spoilage and pathogenic microorganisms. Metabolic products of LAB exhibit a wide range of antimicrobial properties. Lethal action by organic acids is related to their dissociation constant (pKₐ) (4.75 for acetic acid, and 3.86 for lactic acid) (Adams and Moss 2008), and will therefore be dependent on the pH level reached during fermentation. Lactic acid—the major metabolite in homofermentation—will inhibit or destroy putrefactive and gram-negative bacteria and some fungi, while putrefactive bacteria, numerous gram-negative bacteria, clostridia, some yeasts, and fungi will be inactivated by acetic acid, produced during heterofermentation in addition to lactic acid. Hydrogen peroxide is toxic to many pathogens and spoilage organisms, especially in protein-rich foods. Low molecular weight metabolites such as reuterin (3-OH-propionaldehyde) may be produced only by particular strains, and show potent antimicrobial action against a wide spectrum of bacteria, molds, and yeasts. Diacetyl, associated with the desired “butter aroma” in fermented dairy products (but unacceptable in beer), will inhibit gram-negative bacteria. Fatty acids, resulting from mild lipolytic activities, are inhibitory or toxic to different bacteria. Bacteriocins are antimicrobial substances of a proteinaceous nature that are active against closely related bacteria.
They exhibit a narrow spectrum of activity but are generally not considered to be active against gram-negative bacteria. A low frequency (from 0.6% to 22%) of bacteriocinogenic strains may be expected among food-associated LAB (Holzapfel 1997, 2002). The frequency of bacteriocinogenic LAB in African fermented foods appears to lie within the same range (Olasupo et al. 1994). Ben Omar et al. (2008) showed that 31 of 135 (22.9%) isolates, mostly *Lactobacillus plantarum*, from the Congolese fermented maize product *poto poto*, produced bacteriocin. In our study on LAB from cassava fermentation in the production of *gari*, it was determined that 12% of heterofermentative cocci, mostly *Leuconostoc fallax* strains, and 63% of facultative heterofermentative rods, mostly *L. plantarum* strains, were presumptive bacteriocin producers (Kostinek et al. 2005). The special interest in bacteriocinogenic LAB is based on their possible application in food safety assurance (Klaenhammer 1993; Schillinger et al. 1996; Adams and Nicolaides 1997). Moreover, they may play an important role in amplifying the safety of food fermentsations. Bacteriocinogenic LAB have been shown to effectively inhibit the growth of pathogens, such as *Listeria monocytogenes*, *Staphylococcus aureus*, *Bacillus cereus*, and *Clostridium difficile*, even under *in situ* conditions (Holzapfel et al. 1995).

Investigations on beneficial interactions of fermented foods with the GIT have also clearly pointed to lactic-fermented foods and their “natural” population of putative functional LAB. Studying the underlying mechanisms of beneficial “probiotic” effects in the GIT and its autochthonous microbiota presents a challenging and exciting field of multidisciplinary research, both for gastroenterologists, molecular biologists, microbiologists, food scientists, and human physiologists.

Fermented foods still play a major role in the diet of numerous traditional societies worldwide. The typical characteristics of these foods vary according to tradition, region, availability of raw materials, and taste preferences. For example, it appears that (lactic) fermented meat such as salami is not generally appreciated in most regions of Africa. On the other hand, the lactic fermentation of cereals in particular enjoys wide acceptance throughout the African continent, but to a lesser extent in Asia, where (mainly nonlactic) fermentation of legumes dominates. According to Tamang and Samuel (2010), the world dietary culture has three distinct traditional food habits based on staple cereal diets: (a) cooked-rice eaters of Eastern food culture, (b) wheat/barley-based breads/loaves of Western and Australian food culture, and (c) sorghum/maize porridges of African and South American food culture. The African dietary culture includes both fermented and nonfermented sorghum, maize, millets, cassava products, wild legume seeds and tubers, but also meat, milk products, and alcoholic beverages (Tamang and Samuel 2010). Interestingly, some major cereal crops, in particular maize—the most widely used raw material for lactic fermentation in Africa—have their origins in Mexico and Latin America, and were probably brought to Africa by Portuguese vessels during the 17th century or earlier (Burtt-Davy 1914). Traditional fermented products based on maize were developed by the indigenous populations of Mexico and Peru in the pre-Colombian era, and have been consumed in the fermented form for hundreds of years (Haard et al. 1999). These fermented products are still of importance, especially for rural communities, in Latin America.

There are about 5000 varieties of major and minor unlisted fermented foods and beverages in the world prepared and consumed by billions of people. Chinese, Indians (several tribes) from Latin America, and Africans (several tribes) have the largest variety of ethnic fermented foods and beverages (Tamang 2010). The vast range and diversity of traditional fermented foods worldwide cannot be effectively summarized in this chapter, and reference will only be made to a few examples, some of which present interesting models of safe and functional traditional foods, thanks to “upgrading” by fermentation.
14.2 African Continent and Regional Product Specificities

Of importance to all rural societies, globally, are low-cost and, where possible, “low-tech” food processing procedures affordable also by the poor. Since ages, food fermentation is indigenous to African cultures and still plays a major role in combating food spoilage and foodborne diseases, which are prevalent in many underdeveloped regions. Lactic acid fermentation is probably the oldest and best accepted among the African peoples (Dirar 1993), and is largely a home-based process throughout the African continent (Oyewole 1997).

In terms of fermented foods, the African continent is characterized by the domination of lactic fermentations of predominantly cereals. The lactic fermentation technology has developed to include a wide range of raw materials, yielding an impressive range of products (Oyewole 1997), most of which are relatively safe, even for use by infants. In Table 14.1, examples of fermentation types and raw materials are given, exemplifying the diversity of lactic food fermentations in Africa. A more detailed listing of the foods and the microorganisms involved in the fermentation is shown in Table 14.2. In contrast to the Asian and European continents, fish, meat, and vegetables are not typical raw materials for fermentation in Africa. On the other hand, a variety of cereals and root crops serve as typical raw materials for lactic fermentation in most regions of Africa (Table 14.2).

Table 14.1 Examples of the Diversity of Food Fermentations in the African Continent

<table>
<thead>
<tr>
<th>Categories</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
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</thead>
<tbody>
<tr>
<td>ogi</td>
<td></td>
<td>kenkey</td>
<td>dawadawa</td>
<td>palm wines</td>
<td></td>
</tr>
<tr>
<td>uji</td>
<td></td>
<td>uji</td>
<td>soumbala</td>
<td>injera</td>
<td></td>
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<tr>
<td>mawe</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>injera</td>
</tr>
<tr>
<td>mahewu</td>
<td></td>
<td></td>
<td>ugba</td>
<td>traditional beers</td>
<td></td>
</tr>
<tr>
<td>gari</td>
<td></td>
<td></td>
<td>ogiri</td>
<td>tej</td>
<td>kisra</td>
</tr>
<tr>
<td>kivunde</td>
<td></td>
<td></td>
<td>okpehe</td>
<td>sershbote</td>
<td>kocho</td>
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<tr>
<td>kule naoto</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>kishk</td>
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<tr>
<td>ergo</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>hulumur</td>
<td></td>
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</tbody>
</table>

Note: All categories represent fermentations where LAB either dominate (A, B, and E) the fermentation, or may typically constitute a minor group (C) or a major group together with yeast fermentation (D).

Raw materials: 1 = maize; 2 = millet; 3 = sorghum; 4 = cassava; 5 = legumes; 6 = tef; 7 = wheat; 8 = vegetables; 9 = ensete; 10 = milk; 11 = water melon seeds; 12 = honey; 13 = palm juice; 14 = dates; 15 = bananas.

A = single-step LAB fermentation; B = soaking followed by lactic fermentation; C = alkaline, bacterial fermentation; D = lactic/alcoholic fermentation; E = dough with LAB dominant.
Table 14.2  African Fermented Food Products and Microorganisms Associated with Fermentation (Excluding Alcoholic Beverages)

<table>
<thead>
<tr>
<th>Product</th>
<th>Area of Production</th>
<th>Fermentable Substrate</th>
<th>Microorganisms Reported to Be Involved in Fermentation</th>
</tr>
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<tr>
<td><strong>African, Nonalcoholic Cereal-Based Foods</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Mahewu (magou)</td>
<td>South Africa</td>
<td>maize, sorghum, or millet</td>
<td><em>L. delbrueckii</em> subsp. bulgaricus; <em>L. delbrueckii</em> subsp. delbrueckii; Leuconostoc spp.; heterofermentative lactobacilli</td>
</tr>
<tr>
<td>Ogi</td>
<td>Nigeria, Benin</td>
<td>maize, sorghum, or millet</td>
<td><em>P. pentosaceus</em>, <em>L. fermentum</em>, <em>L. plantarum</em>, yeast (<em>S. cerevisiae</em>, Candida krusei)</td>
</tr>
<tr>
<td>Koko and kenkey</td>
<td>Ghana</td>
<td>maize, sorghum, or millet</td>
<td><em>W. confusa</em>, <em>L. fermentum</em>, <em>L. salivarius</em>, <em>L. vaccinostercus</em>, <em>L. pantheris</em>, Pediococcus spp. and yeast</td>
</tr>
<tr>
<td>Uji</td>
<td>East Africa</td>
<td>maize, sorghum, or millet</td>
<td><em>L. plantarum</em>, <em>L. paracasei</em>, <em>L. fermentum</em>, <em>L. buchneri</em>, <em>P. acidilactici</em>, <em>P. pentosaceus</em></td>
</tr>
<tr>
<td>Kisra</td>
<td>Sudan</td>
<td>sorghum</td>
<td>LAB</td>
</tr>
<tr>
<td>Hussuwa</td>
<td>Sudan</td>
<td>sorghum</td>
<td><em>L. fermentum</em>, <em>P. acidilactici</em>, Ent. faecium (minor proportions)</td>
</tr>
<tr>
<td>Injera</td>
<td>Ethiopia</td>
<td>sorghum</td>
<td>Candida guillermondii</td>
</tr>
<tr>
<td>Ting</td>
<td>Botswana, South Africa</td>
<td>sorghum</td>
<td><em>L. fermentum</em>, <em>L. plantarum</em>, <em>L. rhamnosus</em></td>
</tr>
<tr>
<td>Obusera</td>
<td>Uganda</td>
<td>millet</td>
<td>LAB</td>
</tr>
<tr>
<td>Mawe</td>
<td>Benin</td>
<td>maize</td>
<td>Lact. lactis, <em>P. pentosaceus</em>, <em>L. plantarum</em></td>
</tr>
<tr>
<td>Kuna zaki</td>
<td>Nigeria</td>
<td>millet, sorghum</td>
<td><em>L. fermentum</em>, <em>P. pentosaceus</em>, W. confusa, Ent. faecalis</td>
</tr>
<tr>
<td>Bogobe</td>
<td>Botswana</td>
<td>sorghum</td>
<td>Unknown</td>
</tr>
<tr>
<td>Dégué</td>
<td>Burkina Faso</td>
<td>millet</td>
<td><em>L. gasseri</em>, <em>L. fermentum</em>, <em>L. brevis</em>, <em>L. casei</em>, Enterococcus spp.</td>
</tr>
</tbody>
</table>

(continued)
### Table 14.2 African Fermented Food Products and Microorganisms Associated with Fermentation (Excluding Alcoholic Beverages) (Continued)

<table>
<thead>
<tr>
<th>Product</th>
<th>Area of Production</th>
<th>Fermentable Substrate</th>
<th>Microorganisms Reported to Be Involved in Fermentation</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>African Fermented Starchy Root Products</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gari</td>
<td>West Africa</td>
<td>cassava</td>
<td><em>L. plantarum, Leuc. fallax, L. fermentum</em> (predominate) <em>W. paramesenteroides, L. brevis, Leuc. pseudomesenteroides</em> (minor proportions), <em>Strep. lactis, Geotrichum candidum</em>, <em>Corynebacterium manihot</em> (also reported)</td>
</tr>
<tr>
<td>Lafun</td>
<td>Nigeria</td>
<td>cassava</td>
<td><em>L. fermentum, L. plantarum, W. confusa, yeast</em> (<em>S. cerevisiae, Pichia scutulata, Klyveromyces marxianus, Hanseniaspora guillermondii</em>), and <em>Bacillus</em> spp.</td>
</tr>
<tr>
<td>Fufu</td>
<td>Nigeria</td>
<td>cassava</td>
<td><em>P. pentosaceus, L. fermentum, L. plantarum</em></td>
</tr>
<tr>
<td>Kivunde</td>
<td>Tanzania</td>
<td>cassava</td>
<td><em>L. plantarum</em>, other LAB, yeast</td>
</tr>
<tr>
<td>Chikawngue</td>
<td>Zaire</td>
<td>cassava</td>
<td>LAB, yeast</td>
</tr>
<tr>
<td>Cingwada</td>
<td>East and Central Africa</td>
<td>cassava</td>
<td>Unknown</td>
</tr>
<tr>
<td>Kocho</td>
<td>Ethiopia</td>
<td>ensete or Abyssinian banana (<em>Ensete ventricosum</em>)</td>
<td>LAB yeast</td>
</tr>
<tr>
<td>Agbelima</td>
<td>Ghana</td>
<td>cassava</td>
<td><em>L. plantarum, L. brevis, L. fermentum, Leuc. mesenteroides</em>, also <em>Bacillus</em> spp., <em>Candida tropicalis, Geotrichum candidum, Penicillium</em> spp.</td>
</tr>
</tbody>
</table>

### African Fermented Animal Proteins

<table>
<thead>
<tr>
<th>Product</th>
<th>Area of Production</th>
<th>Fermentable Substrate</th>
<th>Microorganisms Reported to Be Involved in Fermentation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nono (milk curd)</td>
<td>Northern part of West Africa</td>
<td>milk</td>
<td>LAB</td>
</tr>
<tr>
<td>Maziwa lala</td>
<td>East Africa</td>
<td>milk</td>
<td><em>Strep. lactis, Strep. thermophilus</em></td>
</tr>
</tbody>
</table>

(continued)
Table 14.2  African Fermented Food Products and Microorganisms Associated with Fermentation (Excluding Alcoholic Beverages) (Continued)

<table>
<thead>
<tr>
<th>Product</th>
<th>Area of Production</th>
<th>Fermentable Substrate</th>
<th>Microorganisms Reported to Be Involved in Fermentation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leban (sour milk)</td>
<td>Morocco</td>
<td>milk</td>
<td>Lactic streptococci (lactococci), Leuc. lactis, Leuc. mesenteroides subsp. cremoris</td>
</tr>
<tr>
<td>Wara</td>
<td>West Africa</td>
<td>milk</td>
<td>Lactococcus lactis, Lactobacillus spp.</td>
</tr>
<tr>
<td>Ergo</td>
<td>Ethiopia</td>
<td>milk</td>
<td>Lactobacillus spp., Lactococcus spp.</td>
</tr>
<tr>
<td>Kule naoto</td>
<td>Kenya</td>
<td>milk</td>
<td>L. plantarum, L. fermentum, L. paracasei, L. acidophilus, also lactococci, leuconostocs, and enterococci</td>
</tr>
<tr>
<td>Sethemi</td>
<td>South Africa</td>
<td>milk</td>
<td>Lactobacilli, lactococci, yeast (Debaromyces hansenii, S. cerevisiae, Cryptococcus curvatus)</td>
</tr>
<tr>
<td>Guedj</td>
<td>Senegal</td>
<td>fish</td>
<td>Lact. lactis</td>
</tr>
<tr>
<td>Bonome (stink fish)</td>
<td>Ghana</td>
<td>fish</td>
<td>Unknown</td>
</tr>
</tbody>
</table>

**African Fermented Vegetable Foods**

<table>
<thead>
<tr>
<th>Product</th>
<th>Area of Production</th>
<th>Fermentable Substrate</th>
<th>Microorganisms Reported to Be Involved in Fermentation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dawadawa or iru, Ogiri, Ogiri-nwan, Ogiri-igba, Ogiri-saro, Ogiri/okpec/ okpehe, Ugba, Owoh, Bukalga</td>
<td>Various African countries and regions</td>
<td>African locust bean, soybean, melon, fluted pumpkin bean, castor oil seed, sesame seed, mesquite, African oil bean, cotton seeds, Kartade, red sorrel</td>
<td>Predominantly Bacillus spp. fermentations</td>
</tr>
</tbody>
</table>


Notes: Ent., Enterococcus; L., Lactobacillus; Lact., Lactococcus; Leuc., Leuconostoc; P., Pediococcus; S., Saccharomyces; Strep., Streptococcus; W., Weissella.
Fermented foods in Africa can be classified based on the fermentable raw material used in their production into the following major groups (Olasupo et al. 2010):

- Fermented nonalcoholic cereals
- Starchy root crops
- Fermented animal proteins
- Fermented vegetable proteins
- Alcoholic beverages

Practically all alcoholic beverages are characterized by the initiation of fermentation by LAB, and particularly *Leuconostoc* spp. such as *Leuc. mesenteroides* subsp. *mesenteroides*, as can be typically observed for palm wine (Holzapfel, unpublished results). Moreover, the first stage, typical of traditional beer fermentation throughout Africa, is dominated by LAB such as *L. debrueckii* subsp. *debrueckii* and mesophilic hetero- and homofermentative lactobacilli (Haggblade and Holzapfel 2004). Due to space limitation, the main focus in this chapter will be on examples of “pure” lactic fermentations (see also Table 14.2).

Milk from cattle, sheep, and goats is typically fermented in Eastern and Southern Africa, and some regions in North Africa, where keeping of cattle, sheep, and goats has a long tradition. Fermentation of camel’s milk has been practiced since ancient times in Northern Africa and the Sudan. Indigenous legumes such as the locust bean, mesquite, but also soy, serve as raw materials for an “alkaline” fermentation dominated by *Bacillus subtilis* for producing of a condiment (*soumbala, dawadawa, okpehe*) typical of West Africa (Table 14.2). This product has similarity to *kinema*, a condiment typical in the Himalayas produced from soybeans (Sarkar et al. 2002) from which enterococci can be isolated. This was shown for okpehe fermentations, but the presence of elevated numbers of enterococci may cause an uncharacteristic “cheese-like” flavor (Oguntoyinbo et al. 2007, 2010). Another problem with the alkaline food fermentations in which *Bacillus* spp. predominate is the occurrence of the foodborne pathogen *B. cereus*. Toxinogenic *B. cereus* strains were reported to occur in African fermented foods such as okpehe (Oguntoyinbo et al. 2010); *iru* and *ogiri* (Oguntoyinbo and Oni 2004); and *afitin, iru*, and *sonru* (Thorsen et al. 2010). Also typical for West African tradition are the root-crop fermentations, mainly using cassava (*Manihot esculenta* Crantz), although the detoxification processing of bitter cassava by spontaneous lactic fermentation can be found throughout the African continent (Okafor and Ejiofor 1990; Westby 2002; Ray and Sivakumar 2009).

Specific of Ethiopia is the *ensete* (*Ensete ventricosum*) (also called false banana, Ethiopian banana, or Abyssinian banana) plant, which, according to the Food and Agriculture Organization (FAO), provides a higher food crop per unit area than most cereals, and may supply enough food for a family of five to six people. It can be converted into *kocho* by lactic fermentation (Gashe 1987).

Maize (corn), sorghum, and millet are most commonly fermented throughout Africa, while tef (*Eragrostis tef*), typical of Ethiopia, is used for preparing the traditional acid-leavened (sourdough) type of “pancake” called *injera* (Oyewole 1997; Urga et al. 1998). In contrast to Asia and Europe, rice and wheat are only rarely being used for fermentation in the African continent (Oyewole 1997).

### 14.3 Nonalcoholic Cereal Fermentations

Cereals account for up to 80% of total caloric consumption in many African countries and are an important source of dietary protein in most of these countries. The major part of traditional
African cereal-based foods are processed by natural fermentation, and frequently play a key role as weaning foods for infants and as dietary staples for adults. Lactic-fermented cereal-based foods in Africa can be classified on the basis of either the raw cereal ingredients used for preparation, or the texture of the fermented product (Oyewole 1997; Haard et al. 1999; Nout 2009).

**According to raw cereal ingredients (with country or region):**

- **Maize-based foods:** *ogi* (Nigeria/West Africa), *kenkey* (Ghana), *mawe* (Benin)
- **Millet-based foods:** *kunuzaki* (Northern Nigeria), *mbege* (Tanzania), *ben-saalga* (Burkina Faso)
- **Sorghum-based foods,** for example, *ogi* (West Africa), *bogobe* (Botswana), *humulur* (Sudan), *hussuwa* (Sudan); effervescent or “opaque” beers (throughout Africa), for example, *pito* (Ghana), *tcchoutou* (Benin), and *burukutu* (Nigeria)
- **Wheat-based foods:** *bouza*, *kishk*, or *kishj* (Egypt)

**On the basis of texture:**

- **Liquid or gruel:** *ogi* (Nigeria), *mahewu* (Southern Africa), *burukutu* (Nigeria), *pito* (Ghana), *uji* (Kenya)
- **Solid-state fermentation (dough) and dumplings:** *kenkey* (Ghana), *agidi*
- **“Dry” flatbread or pancake-like:** *kisra* (Sudan), *injera* (Ethiopia)

### 14.3.1 Nutritional Benefits of Lactic Fermentation of Cereals

Antinutritive components are of particular significance in “unbalanced” diets rich in starch, based on cereals such as maize, in which the amino acid score of the protein is low due to a deficiency in essential amino acids such as lysine, tryptophan, and methionine. This can be compensated by consuming starchy staple foods (maize, sorghum, and millet) in combination with fish or legumes such as soya or cowpeas (Nout and Motarjemi 1997). These combined products, however, may contain several antinutritive factors, including protease and amylase inhibitors, polyphenols (from millets and sorghum), and lectin (associated with hemagglutinin activities in legumes and tannins). These factors may reduce the protein and starch availability, while the bioavailability of minerals such as calcium, iron, magnesium, and zinc may be reduced owing to the chelating properties of phytic acid (Holzapfel 1997, 2002; Nout 2009). Antinutritional factors, coupled with the lysine, tryptophan, and methionine deficiencies, in cereal proteins contribute to malnutrition in developing countries (Holzapfel 1997). The nutritional value of cereal staples can significantly be improved by fermentation, which may serve to reduce several antinutritive factors (Chavan and Kadam 1989; Mbugua et al. 1992; Lorri 1993; Nout and Motarjemi 1997). LAB strains, however, differ in their ability to degrade trypsin inhibitor. Under defined conditions, approximately 50% reduction in trypsin inhibitor activity was observed in *L. plantarum* strain 91 and *Leuconostoc* sp. 106 isolated from Ghanaian fermented foods (Holzapfel 1997). Soaking can activate endogenous phytases in most cereals and legumes, while lactic fermentation may also contribute to reduce the phytate content of white sorghum (Svanberg and Sandberg 1988), non-tannin-containing cereals (Svanberg et al. 1993), maize (Lopez et al. 1983), and pearl millet (Khetarpaul and Chauhan 1989). Phytates have been found to be reduced noticeably during fermentations (Svanberg and Sandberg...
Lactic Acid Bacteria: Microbiological and Functional Aspects

The phytic acid–degrading ability is relatively rare among pure LAB cultures, but some *L. plantarum* and *L. pentosus* strains have been found capable of degrading phytic acid (Holzapfel 1997; Palacios et al. 2005; Songre Ouattara et al. 2008). Moreover, raffinose, stachyose, and verbascose are oligosaccharides that typically occur in legumes and cereals, and which may cause flatulence, diarrhea, and indigestion in humans. These sugars possess α-d-galactosidic bonds resistant to cooking and small-scale processing, but which can be hydrolyzed by α-galactosidases produced by a number of molds and by bacteria associated both with the digestive tract and fermented foods. Extensive studies using both pure and mixed cultures have shown variable abilities of strains of several LAB species to produce α-galactosidase, for example, *Leuc. mesenteroides* subsp. *mesenteroides* and subsp. *dextranicum* and *Weissella paramesenteroides* (Milliere et al. 1989). However, it appears to be a constitutive property of *L. fermentum*, *L. brevis*, *L. buchneri*, and *L. salivarius* (Mital et al. 1973), and was suggested to be inducible in *L. plantarum* ATCC 8014 (Ahrne and Molin 1991). *L. plantarum* strains isolated from fermented Ghanaian maize products were able to ferment raffinose, but not strains of *Pediococcus acidilactici* and *P. pentosaceus* (Holzapfel 2002). More than 70% of *L. plantarum* and *L. fermentum* strains from fermented cassava in the production of gari were shown to ferment raffinose, while about 20% of *L. fermentum* and *Leuc. fallax* strains were capable of stachyose fermentation (Kostinek et al. 2005).

A mortality rate of up to 5 million per year, due to foodborne infections, is estimated for children under the age of 5 years in developing countries. Safety and health effects of lactic-fermented cereal gruels over those of nonfermented foods from the same raw materials have shown clear beneficial effects in several studies and surveys. In a study conducted in Tanzania by Lorri and Svanberg (1994) (also published online: http://unu.edu/unupress/food/8F151e/8F151E0b.htm), a lower prevalence of diarrhea in young children fed lactic acid–fermented cereal gruels was statistically proven. Two groups of about 100 children under 5 years of age were selected on the basis of the use or non-use of fermented gruels. The nutrition status of the children in each group, as measured by comparison of their weight for age with the standard, was not statistically different. The mean age of the children in the two groups was similar. An average feeding frequency of 3.5 times per day was reported for both groups. Biweekly recall for diarrhea, defined as watery, loose stools two or more times per day, was conducted for 9 months among selected children. The mean number of diarrhea episodes over the study period was 2.1 for children eating fermented gruels, compared with 3.5 for those eating nonfermented gruels. The frequency of diarrhea was also age dependent: it was significantly higher in children under 3 years of age than in older children in both diet groups. Fever episodes were reported in similar proportions in both groups, and were significantly related to diarrhea. Other diseases and the children’s nutrition status were not related to the frequency of diarrhea (Lorri and Svanberg 1994).

A similar study, also conducted in Tanzania (Kingamkono 2003), showed that, compared with the baseline, the prevalence of pathogenic fecal bacteria was reduced during the study period in children consuming lactic acid–fermented cereal gruel. Age-matched healthy children from a local maternal and child health clinic were used as controls. Moreover, children in the cereal gruel group showed a faster drop in lactulose-to-mannitol (L/M) permeability ratio between admission day and day 3 compared with the other two study groups. The L/M ratio on day 3 was also lower than in the other two groups.

A pure lactic-fermented maize gruel that is well known and appreciated throughout Southern Africa is mahewu (Xhosa: *amarewu*, Zulu: *amabewu*, Swazi: *emahewu*, Sotho: *machleu*). It has been industrialized, one process being a thermophilic fermentation and another a mesophilic “mixed” strain fermentation. Yet very little has been published on the exact taxonomy of typical
LAB associated with mahewu fermentation. The standardized product contains 8–10% solids and has a pH of about 3.5 (titratable acidity 0.4–0.5%). Typical of the traditional fermentation are *Leuc. mesenteroides* and *L. brevis*, although *L. delbrueckii* has also been reported to be associated with mahewu (Holzapfel 1989).

### 14.3.2 Examples of Fermented Maize

Mawe is an uncooked fermented maize dough, and serves as an important ingredient for the preparation of cooked bread, stiff gels, and steamed cooked bread in Benin (Nout 2009). It is prepared by “natural” (uncontrolled) fermentation, which may be supported by “recycling” or “backslopping” of microorganisms in support of a relatively stable (predictable) process, dominated by heterofermentative LAB such as *L. fermentum, L. brevis, L. curvatus, L. buchneri, W. confusa*, and pediococci (Hounhouigan et al. 1993). Typical of most traditional lactic fermentations, yeasts such as *Candida krusei, C. kefyr, C. glabrata*, and *Saccharomyces cerevisiae* may also play some role in the fermentation (Hounhouigan et al. 1994; Nout 2009). Fermentation promotes the formation of acidity and flavor. In addition, it enhances digestibility, the supply of macronutrients such as carbohydrates and some proteins, and also increases the bioavailability of micronutrients, such as minerals and B vitamins.

Other examples of fermented maize foods include the Nigerian ogi, the Kenyan uji, and the Ghanaian kenkey, although variations occur in procedures, and because millet and sorghum can also be used as raw fermentation materials for the former two (Oyewole 1997; Nout 2009). The pH is reduced by the fermentation process to below 4.0, thereby inhibiting most spoilage organisms naturally present in maize, sorghum, or millet. The acidity of fermented uji was shown to correlate with increased shelf life and to contribute to hygienic safety (Mbugua and Njenga 1992). Preparation of both kenkey and fermented uji involves soaking of the grains until soft, wet grinding, and fermentation.

Fermentation of uji differs from that of kenkey by the slurrying and removal of coarse particles and bran by filtration, followed by fermentation of the filtrate (Onyango et al. 2004). The fermentation of uji during sedimentation is predominated by LAB such as *L. plantarum, L. fermentum, L. cellobiosus, L. buchneri, P. acidilactici*, and *P. pentosaceus* (Mbugua 1985).

Two types of kenkey are produced, *fanti-* and *ga-kenkey*, with differences in salt content and packaging material (Nout et al. 2007). Following soaking and wet milling, half of the mass is kneaded with some water into a dough that ferments for 3–4 days, and then the other half is mixed with water and cooked to obtain a gelatinized mass called aflata. Equal quantities of aflata and fermented dough are mixed and kneaded into a sticky dough that is molded into balls, which are wrapped in either plantain or maize leaves, and cooked in water for several hours. The final kenkey has a shelf life of several days, and is consumed as “ready-to-eat” meal together with other foods (Nche 1995; Nout 2009). Dominant microorganisms are *L. plantarum, L. fermentum, L. brevis, L. reuteri*, and *P. pentosaceus* (Olsen et al. 1995), as well as yeasts, mainly *C. krusei (Issatchenkia orientalis)* and *S. cerevisiae* (Jespersen et al. 1994).

### 14.3.3 Examples of Fermented Sorghum

Hussuwa is a semisolid, dough-like food of the Sudan, made by the fermentation of sorghum or millet malt and an “aceda” (stiff porridge from unmalted flour) on a small-scale, home-production level. A liquid paste of sorghum flour and water are mixed in equal volumes, cooked to the stiff aceda porridge, and a further half volume of sorghum malt added and left to ferment for up to
48 h. The resulting sourdough, called “ajin,” is cooked on a hot plate until all moisture is expelled (El-Nour et al. 1999; Yousif et al. 2005). After cooking, the hussuwa is fermented in an earthenware pot buried under the fireplace for up to 2 months, thereby ensuring a continuous warm temperature throughout the period to promote mixed lactic and alcoholic fermentation (Yousif et al. 2005), finally resulting in a sweet–sour product (Dirar 1993). Due to the small-scale level and typical spontaneous fermentation, the product quality and safety may vary. It is interesting that enterococci, particularly Enterococcus faecium strains, constitute about 10% of the LAB strains isolated from hussuwa (Yousif et al. 2005). A polyphasic taxonomic study showed that the majority (58%, 128 out of 220) of the LAB isolates typical of hussuwa fermentation were heterofermentative lactobacilli, of which 97 of 100 isolates were characterized as L. fermentum. Of the 220 isolates, 72 (32.7%) were pediococci, and 92.7% of the 41 selected isolates were characterized as P. acidilactici, and the rest as P. pentosaceus. A selection of L. fermentum and P. acidilactici were investigated for their use as starter cultures (Yousif et al. 2010) and random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) fingerprinting showed they could establish themselves and predominate in the fermentation. The use of starter cultures furthermore led to a rapid decrease of pH to below pH 4.0 in 48 h.

14.3.4 Examples of Fermented Millet

Ben-saalga, a thin beverage, is prepared by cooking the fermented sediment of pearl millet (Pennisetum glaucum) in water, and is typical of Burkina Faso (Nout 2009). L. fermentum, L. plantarum, and P. pentosaceus typically dominate the natural fermentation. As some L. plantarum strains are able to degrade starch (Ben Omar et al. 2006; Sangre Ouattara et al. 2008), considerable losses of millet nutrients may occur, also as a result of discarding the coarse particles, and by leaching into the water fraction. However, antinutritional components of pearl millet such as phytate were reported to be degraded by more than 50% in commercial ben-saalga, thereby facilitating the dietary uptake of proteins and minerals (Lestienne et al. 2005; Nout 2009).

14.4 Starchy Root Crop Fermentations

Worldwide, more than 500 million people rely on cassava as their major daily energy source (Cock 1985; Moorthy and Mathew 1998), with around 200 million living in sub-Saharan Africa (Kimaryo et al. 2000). In Africa, cassava is the major root crop processed by lactic fermentation, providing a wide range of safe products such as gari, fufu, and lafun or kokonte, common throughout West Africa, and kivunde and cingwada in East Africa (Kimaryo et al. 2000). The shelf life of cassava is less than 5 days as it starts to deteriorate shortly, 24 h after harvesting. Use of lactic fermentation to yield the above-mentioned products drastically prolongs the shelf life, and, for bitter cassava, improves the toxicological safety. Cassava grows in poor and acidic soils often not suitable for other crops, and yield a harvest even in times of drought when most other crops have failed to grow because of lack of water (Mlingi 1995), and thus has replaced sorghum as the main staple in some parts of Tanzania and other regions in Africa since its introduction at the beginning of the 20th century (Mlingi et al. 1992, 1993). While sweet varieties of cassava may be consumed directly, bitter varieties are traditionally processed into a wide variety of products with different local names (Amoa-Awua 1996). The cyanide in cassava occurs as cyanogenic glucosides, mostly 2-(β-d-glucopyranosylxoyl) isobutyronitrile (linamarin) and, to a lesser extent, its derivative 2-(β-d-glucopyranosyloxy) methylbutyronitrile (lotaustral). Different processing methods,
including fermentation, can be used for detoxification. \textit{L. plantarum} typically dominates the fermentation process and was shown also to be inhibitory to many spoilage molds and bacteria, both in the East African (Tanzanian) (Kimaryo et al. 2000) and West African (Ghanaian) fermentation process (Amoa-Awua et al. 1996). In a study conducted on cassava in Benin, \textit{L. plantarum} was the most abundantly isolated species (54.6\% of isolates), followed by \textit{Leuc. fallax} (22.3\%) and \textit{L. fermentum} (18.0\%), with “minorities” of \textit{L. brevis}, \textit{Leuc. pseudomesenteroides}, and \textit{W. paramesenteroides}, which were only sporadically isolated (Kostinek et al. 2005). In a controlled study, selected \textit{L. plantarum} strains showed potential as starter cultures for cassava fermentation in the kivunde process, during which the residual cyanide levels after 120 h fermentation were below the maximum value of 10 mg/kg recommended by Codex/FAO for cassava flour. The effect of the \textit{L. plantarum} strains was suggested to be related to their glucosidase activity, and points to the potential of selected starter cultures for the improvement of traditional African fermented foods such as kivunde (Kimaryo et al. 2000). Two LAB strains, \textit{L. plantarum} BFE 6710 and \textit{L. fermentum} BFE 6620, isolated from cassava fermentations in Benin, were used to start cassava fermentations in a pilot study under field production conditions in Kenya. Using RAPD-PCR and pulse-field gel electrophoresis strain typing techniques showed that \textit{L. plantarum} BFE 6710 was successfully established as a predominant strain during fermentation, while \textit{L. fermentum} BFE 6620 showed weaker survival ability (Kostinek et al. 2008). With the purpose of selecting appropriate starter cultures for cassava fermentation for gari production, the ability of lyophilized LAB strains to recover their acidification activity was evaluated through the determination of the pH, titratable acidity (% lactic acid/g dry mass), and cell count over 24 h, and strains of \textit{L. plantarum}, \textit{L. fermentum}, and \textit{W. paramesenteroides} were found suitable for distribution and application as freeze-dried starter cultures for gari production (Yao et al. 2009). Indeed, freeze-dried cultures were used to start gari fermentations in a Hazard Analysis Critical Control Point–built rural pilot plant in Quedo village in Benin run by a women’s group of 13 members. The use of the starter cultures resulted in rapid acidification of the mash, ensuring a high safety of the final product, and in a reduction of the fermentation period from 24 to 12 h (Egounlety et al. 2007).

### 14.5 Animal Protein Fermentations

In Africa, animal protein fermentations are mostly related to milk fermentations, although some fish fermentations also occur. Keeping of livestock with concomitant milk production has a long tradition in East and Southeast Africa, where the utilization of fermented milk products is frequently integrated into the culture, comprising an important part of the daily diet. Ethiopia has probably the largest livestock population in sub-Saharan Africa and figures among the top 10 in the world, with surveys reporting 27 million head of cattle, 24 million sheep, 18 million goats, 7 million equines, 1 million camels, and 52 million poultry (World Health Organization 1996; Gonfa et al. 2001). The four main sources of milk Ethiopia are local zebu cattle, cross-breed cattle (mainly with Friesian and Holstein), and pure- or nearly pure-breed Friesian or Holstein cattle on the state farms. Milk fermentation is mainly by traditional, small-scale “sour milk technologies” in rural areas to convert milk into various products for extending shelf life. The major fermented milk products produced in Ethiopia by smallholder farmers using traditional methods are (Gonfa et al. 2001):

- \textit{Ergo} (fermented sour milk)
- \textit{Ititu} (fermented milk curd)
- \textit{Kibe} (traditional butter)
Relatively little is known about the microbiology and presumed dominating LAB of these fermentations. A microbiological study of traditionally processed ititu showed that it contains high microbial numbers of lactobacilli with *L. casei* and *L. plantarum* as the dominating species (Kasseye et al. 1991; Gonfa et al. 2001). *Ayib* contributes to the overall nutrition of the people and forms part of the staple diet; little is known about the typical LAB population, but lactose-assimilating yeasts were found to play a role in the fermentation in addition to LAB, which also included enterococci, with a total population of up to $10^8$ cfu/g (Ashenafi 1990; Gonfa et al. 2001).

Traditions in dairy fermentations are partly comparable between Ethiopia and its neighbor states, Kenya and Sudan, and, to some extent, Tanzania and Uganda. Of special interest is the traditional Maasai fermented milk, *kule naoto*, because of its importance as the major daily diet of the Maasai community in Kenya and Tanzania. The Maasai people rarely consume fruits or grains, while their standard daily diet comprises on average 2–3 liters of the fermented milk per person. Kule naoto is produced from unpasteurized whole milk from zebu cows using centuries-old tribal practices in the rural areas for “spontaneous” fermentation in a calabash for at least 5 days. Kenyans appreciate the product for its excellent natural taste and aroma. In addition they ascribe functional benefits to consumption of kule naoto because of a therapeutic value for protection against diarrhea and constipation (Mathara 1999). In a study on 300 LAB isolates from Kenyan traditional kule naoto, the genus *Lactobacillus* was found to dominate the fermentation, followed by *Enterococcus*, *Lactococcus*, and *Leuconostoc*. Interestingly, *L. plantarum* (60%) was shown to be the dominating species, with numbers ranging from $10^7$ to $10^8$ cfu/ml. *L. fermentum*, *L. paracasei*, and the *L. acidophilus* “group” were detected at a level of $10^6$ to $10^8$ cfu/ml (Mathara et al. 2004).

### 14.6 Quest for Probiotic African Fermented Foods

In the Western dietary culture, milk fermentations into yogurt, sour milk, or cheese are common and have a huge market and dietary impact. Not surprisingly, from a background of Metchnikoff’s studies on the health properties of yogurt, probiotics are predominantly associated with fermented milks, particularly sour milk and yogurt products in Western countries. As seen above, animal protein fermented foods, including milk fermentations, are not the major fermented foods in Africa. On the other hand, in those regions (e.g., Ethiopia, Kenya) where milk is fermented and consumed regularly, the production of foods containing probiotics makes sense. However, such products would probably not find wide distribution across the African continent. In addition, the marketing of Western probiotic milk products would be unpractical because of the unfamiliarity and relatively high costs of such products in the poor regions of Africa. Africa is, however, a continent where there is high incidence of HIV and diarrhea (Reid et al. 2005), especially among young children, and where child mortality of children less than 5 years old is extremely high. Thus, the utilization of fermented foods containing probiotics would be one avenue to improve the health of children.

In Africa, probiotic foods should clearly rely on traditional fermented foods, probably well suited for this are the nonalcoholic cereal types of foods that are not heated after fermentation. As seen above, the predominant LAB associated with such foods are lactobacilli and pediococci. Among the lactobacilli, *L. plantarum* and *L. fermentum* strains are particularly predominant in many fermentations. As a well-known *L. plantarum* strain WCFS1 is already utilized in Europe as
a probiotic, and since the species is known to be associated with the human gastrointestinal system, this species may also be a good choice for development as a probiotic in African fermented foods.

To be utilized successfully as a probiotic, the strains used should possess functional properties related to their health-improving activity, and they should optimally also be able to be used as a starter for the fermentation, ensuring that they predominate in the fermentation and can be consumed in high numbers. Thus, the development of such a probiotic demands an extensive investigation into the functional properties of the strain (e.g., gastrointestinal survival, colonization of the GIT, enhancement of the immune response), as well as technological properties (such as possibility to be produced at low cost; adaptation to the substrate and competition with the autochthonous microbial populations, capable of fast acid production to low levels; good viability/survival in the product, also during storage; and the capability to break down antinutritive factors associated with the raw material) would be of great advantage.

Several studies on LAB from the Maasai fermented milk product kule naoto aimed to investigate the potential of the predominant bacteria for development of probiotics. In studies on their functional properties, strains of the *L. acidophilus* group, *L. paracasei*, *L. plantarum*, and *L. rhamnosus*, showed resistance to gastric juice and bile, while some expressed bile salt hydrolase activity and the ability to assimilate cholesterol *in vitro*. *In vitro* adhesion to HT29 MTX cells of up to 70% was recorded, and also a high survival rate under simulated stomach acidic conditions and physiological bile salt concentrations was assessed (Mathara et al. 2007, 2008). Technological features, such as growth kinetics in fresh heat-treated whole milk medium and survival in the final product during storage at 4°C, were studied for several strains, and *L. acidophilus* BFE 6059, *L. paracasei* BFE 5264, and *Lactococcus lactis* BFE 6049, from Maasai fermented milk, showed potential for use as starters for milk fermentation (Patrignani et al. 2006). Five strains identified as *L. plantarum* and two identified as *L. johnsonii* showed good survival under simulated gastrointestinal conditions, and also showed antimicrobial activity. All strains exhibited bile salt hydrolase activity, while, interestingly, only the *L. plantarum* strains showed β-galactosidase activity (Vizoso Pinto et al. 2006). In further studies, the strain BFE 6128 of the *L. acidophilus* group was identified as *L. johnsonii*, and confirmed to have interesting probiotic properties (Vizoso Pinto et al. 2007, 2009). Moreover, it was studied for modulation of signal pathways involved in innate immunity in enterocytes. It sensitized HT29 intestinal epithelial cells toward recognition of *Salmonella enterica* serovar Typhimurium by increasing the interleukin (IL)-8 levels released after challenge with this pathogen and by differentially modulating genes related to toll-like receptor pathways and innate immunity (Seifert et al. 2010). Other *L. plantarum* strains also showed potential for further development as probiotics as they adhered well to enterocytes and prevented invasion of pathogens into enterocytes in cell culture; they also showed antimicrobial activity toward foodborne pathogens and were able to stimulate IL-8 production of the intestinal epithelial cells *in vitro*.

Lei and Jakobsen (2004) studied the microbiology of the millet porridge *koko* and “koko sour water” (the fermented liquid top layer developing during koko fermentation) produced in Ghana, and proposed the use of koko sour water, which contains approximately $10^8$ live LAB per milliliter, as a probiotic drink. An intervention study based on spontaneously fermented millet as a natural probiotic treatment for diarrhea in young children in northern Ghana was performed by Lei et al. (2006). Children of less than 5 years of age coming to northern Ghana health clinics for treatment of diarrhea were randomized into two groups. Both groups received treatment for diarrhea and in addition the intervention group received up to 300 ml fermented koko sour water daily for 5 days after enrolment. Among the 184 children included, no effects of the intervention could be determined regarding stool frequency, stool consistency, and duration of diarrhea. However, the koko sour water treatment was found to be associated with greater reported well-being 14 days after the
start of the intervention, which may have been due to a preventing effect of the koko sour water on antibiotic-associated diarrhea. Many of the children received antibiotics as a normal part of the treatment, and this was suggested to possibly have masked a probiotic effect associated with the intervention of the koko sour water so that no effect of treatment on symptom alleviation could be determined (Lei et al. 2006).

14.7 Conclusions

As mentioned above, some research efforts into strain development, and investigations into the probiotic potential of fermented foods, have already been conducted. However, more research and input are required on the development of strains with proven functional activity and technological properties in the production of African fermented probiotic foods. There are still major obstacles that hamper such efforts. One example is the lack of industrialization of traditional fermented food processing. Many fermented products in Africa are still being produced on a household scale and then sold at local markets. Even production by small to medium-sized enterprises is rare. Regional variations in raw materials, recipes, and production methods (Nout 2009), and the lack of starter culture technology (Holzapfel 1997), further complicate standardized production. Thus, there is still a considerable need for both research and funding in order to develop African probiotic foods, which, against a background of HIV and diarrheal diseases particularly on this continent, should be of utmost importance. Reid et al. (2005) identified the key steps toward a wider use of probiotics in developing countries to include (a) funding of trials at the local level to assess the applicability to different populations; (b) the introduction of proven products even if the price is not as high as would be attained in the developing world; (c) exchange of students to enhance training and eventually the development of new research centers focused on probiotic foods; (d) introduction of the concepts of probiotics to health-care professionals who are not aware of this field; and (e) connecting with local industries, especially dairies, to provide advice and training on creation, production, and distribution of probiotic foods (Reid et al. 2005). In our view, cereal or starch/cereal mixed fermentations, which have a wider range of occurrence in Africa, should also be seriously considered for the production of fermented foods. The research of probiotic strains and their utilization as multifunctional starter cultures in the production of such fermented foods should be done at research centers in Africa, which are linked to local food processing industry, even if this industry is small or medium sized.

References


Examples of Lactic-Fermented Foods of the African Continent


Haard, N.F., S.A. Odunfa, C.-H. Lee, R. Quintero-Ramírez, A. Lorenecessarily don't divide into sections like this. He doesn't need to set up his work in that way. He just wants to get every piece of information out at once.


Examples of Lactic-Fermented Foods of the African Continent


Chapter 15

Antimicrobial Components of Lactic Acid Bacteria

Ingolf F. Nes, Morten Kjos, and Dzung Bao Diep

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15.1 Introduction

Lactic acid bacteria (LAB) have been used in the household since ancient times owing to their ability to produce and preserve foods. LAB have been shown to be superior to other microorganisms in fulfilling the role of food and feed preservatives, although other organisms have also found a place in more defined products. LAB constitute a diverse group of bacteria that are widely distributed throughout nature, including being an important constituent of the healthy indigenous microflora in humans and animals. Within the LAB group, the genus Lactobacillus is the most widely encountered among probiotics because members of this genus display antimicrobial activities. They produce a number of antimicrobial metabolites, including organic acids, other organic compounds, hydrogen peroxide, and bacteriocins. Food-grade LAB meet the requirements of being ideal microbial food biopreservatives because they have been proven to be nontoxic to humans, they do not alter nutritional properties of the food, they are effective at low concentrations, they are active under refrigerated storage, and they are even considered to possess health-promoting effects. This review will first provide a short description of the low molecular weight, organic antimicrobial metabolite components that are produced by LAB, followed by a more detailed overview of the more complex bacteriocins produced by LAB. Bacteriocins have received considerable interest because of their potent antibacterial activities that apply to the areas of food and health, and also because they are a means of fighting antibiotic-resistant bacteria.

15.2 Low Molecular Weight Organic Compounds with Antimicrobial Activity

15.2.1 Organic Acids

The main principle involved in food and feed preservation by LAB is their ability to produce lactic acid. During growth, the sugars are mostly converted to lactic acid, which exerts most of the inhibitory capacity against microorganisms. When lactic acid is produced, the pH in the fermentate decreases and consequently the organic acids (or small fatty acids, SFA) become undissociated. The undissociated acids (SFAs) are the main antimicrobial feature of LAB. It has been shown that undissociated SFAs target and penetrate bacterial membranes, causing weak acid anions to accumulate in the cytoplasm, thereby affecting metabolic processes. Growth inhibition occurs and the microorganisms eventually die.

Facultative homofermentative LAB also produce acids other than lactic acid (e.g., acetic and formic acids) under certain growth conditions, such as the absence of glucose and the presence of other fermentable carbohydrates. Neither of these acids possesses antimicrobial activities as strong as lactic acid. In addition, other minor antimicrobial metabolites can be produced, such as alcohols and aldehydes, but they are certainly of minor importance for the antimicrobial potency of LAB. In heterofermentative LAB, lactic acids constitute a lower proportion of the acids produced during growth, whereas acetic acid is more abundant.

15.2.2 Reuterin and Reutericyclin

The glycerol-derived antimicrobial compound reuterin has been shown to be produced by a number of lactobacilli. In addition to Lactobacillus reuteri, species such as Lb. brevis, Lb. buchneri
Antimicrobial Components of Lactic Acid Bacteria

Antimicrobial Components of Lactic Acid Bacteria

(Schutz and Radler 1984), Lb. collinoides (Claisse and Lonvaud-Funel 2000), and Lb. coryniformis (Martin et al. 2005; Morita et al. 2008) have also been reported to produce this antimicrobial compound.

Reuterin was initially reported to be produced by Lb. reuteri, which is part of the endogenous bacterial flora in both humans and animals. It is a broad-spectrum antimicrobial agent and is active against gram-positive and gram-negative bacteria, including enteropathogens, yeasts, fungi, protozoa, and viruses. Reuterin is produced under anaerobic conditions, and production is enhanced by the presence of glycerol (Talarico et al. 1988; Lindgren et al. 1990). Reuterin is shown to be an intermediary metabolite involved in the two-step pathway by which glycerol is first dehydrated to form reuterin, some of which is then reduced to 1,3-propanediol (Talarico et al. 1990; Talarico and Dobrogosz 1990; Sriramulu et al. 2008). Its antimicrobial activity against a broad range of microorganisms is attributed to a mixture of monomers, hydrated monomers, and cyclic dimers of β-hydroxypropionic aldehyde.

It has been reported that reuterin is able to inhibit the growth of Aspergillus and Fusarium, and it may therefore play a role in preventing mycotoxin formation in fermented food and feed. Some years ago, a new antimicrobial substance was isolated and named reutericyclin that exhibited a broad antibacterial spectrum, including Lactobacillus spp., Bacillus subtilis, B. cereus, Enterococcus faecalis, Staphylococcus aureus, and Listeria innocua, but it did not target gram-negative bacteria (Gänzle et al. 2000; Höltzel et al. 2000; Gänzle 2004). Reutericyclin was initially referred to as an antibiotic produced by Lb. reuteri that was isolated from sourdough (Höltzel et al. 2000). Reutericyclin was characterized as a negatively charged, highly hydrophobic N-acylated tetramic acid with a molecular mass of 349 Da (Höltzel et al. 2000). The bacteriostatic or bactericidal effect of reutericyclin was attributed to its activity as a proton ionophore on target bacteria, and a broad range of food-related spoilage organisms and pathogens were found to be affected by reutericyclin. It has been suggested that reutericyclin and Lb. reuteri, reutericyclin-producing strains, may have applications in the biopreservation of foods; however, this still remains to be seen.

15.2.3 Antifungal Peptides from LAB

Numerous studies have shown that a large variety of LAB produce antifungal compounds that are different from organic acids; however, they have been difficult to isolate and characterize. This might be because of their low concentrations, chemical composition, diversity and size, low specific activity, stability, being synergistically active molecules with a low individual activity, or other unknown reasons. Schnürer and co-workers were able to isolate and characterize such antifungal peptides from various LAB (Magnusson and Schnürer 2001; Strom et al. 2002; Magnusson et al. 2003; Sjogren et al. 2003; Schnürer and Magnusson 2005). They screened more than 1200 LAB isolates isolated from different environments for antifungal activity. Several isolates inhibited the growth of molds such as Aspergillus species, Penicillium commune, and Fusarium sporotrichioides, as well as growth of the yeast Rhodotorula mucilaginosa. The LAB isolates of interest were identified as Lb. coryniformis, Lb. plantarum, and Pediococcus pentosaceus. Purification showed that the activity found in the growth media could be separated into several active fractions, suggesting a high complexity and diversity of such compounds. Some of the antifungal compounds isolated from an Lb. plantarum strain were identified as cyclo(l-Phe-l-Pro), cyclo(l-Phe-trans-4-OH-l-Pro), and 3-phenyllactic acid (l/d isomer ratio, 9:1) (Strom et al. 2002), and minimal inhibitory concentration (MIC) values against A. fumigatus and P. roquefortii were 20 mg ml⁻¹ for cyclo(l-Phe-l-Pro) and
7.5 mg ml⁻¹ for phenyllactic acid. Combinations of the antifungal compounds revealed weak synergistic effects. The cyclic dipeptides cyclo(l-Phe-l-Pro) and cyclo(l-Phe-traps-4-OH-l-Pro) were also reported to have some antifungal activities. In another study published by the same group, four additional antifungal 3-hydroxyfatty acid compounds were found to be produced by a different isolate of *Lb. plantarum* (Sjogren et al. 2003). The four entities were 3-(R)-hydroxydecanoic acid, 3-hydroxy-5-cis-dodecanoic acid, 3-(R)-hydroxydodecanoic acid, and 3-(R)-hydroxytetradecanoic acid (Sjogren et al. 2003). Racemic mixtures of the saturated 3-hydroxy fatty acids inhibited the growth of several molds and yeasts with MICs between 10 and 100 μg ml⁻¹.

It has been suggested that these chemical compounds could be used as food preservatives; however, further investigations concerning health safety aspects as well as efficacy still have to be assessed. We do not even know whether such antifungal compounds are made in fermented food and feed commodities, or whether they are made in sufficient amounts to exert any antimicrobial effects on fungi, yeasts, or bacteria.

### 15.3 Bacteriocins

Bacteriocins are ribosomally synthesized peptides with antimicrobial activities targeting bacteria closely related to the producer. Gram-positive bacteria and, in particular LAB, seem to produce bacteriocins with a broader target specificity than their counterparts from gram-negative bacteria. This is partly why LAB bacteriocins have received much attention over the last few decades.

The classification of bacteriocins has for a long time been a matter of discussion and disagreement, but there is a general consensus for the two major classes of bacteriocins in LAB, the lantibiotics (Class I) and the nonlantibiotics (Class II). In addition, cyclic bacteriocins have been suggested as comprising their own class and that the peptides/proteins with lytic activity should be grouped in a separate class. Finally, some antimicrobial peptides have been shown to be produced by the specific proteolytic degradation of larger proteins. Numerous reviews on various aspects of bacteriocins have been published in recent years and more details can be found in the literature (Klaenhammer 1988; Jack et al. 1995a; Jack et al. 1995b; Venema et al. 1995; Nes et al. 1996; Saris et al. 1996; Nissen-Meyer and Nes 1997; Baba and Schneewind 1998; Sahl and Bierbaum 1998; Blom et al. 1999; du Toit et al. 2000; Ennahar et al. 2000; Nes and Hole 2000; Cleveland et al. 2001; McAuliffe et al. 2001; Diep and Nes 2002; Hoffmann et al. 2002; Pag and Sahl 2002; Chatterjee et al. 2005; Cotter et al. 2005a; Cotter et al. 2005b; Patton and van der Donk 2005; Bonelli et al. 2006; Drider et al. 2006; Lawton et al. 2007; Willey and van der Donk 2007; Hill et al. 2008; Maqueda et al. 2008; Nissen-Meyer et al. 2009; Papagianni and Anastasiadou 2009; Piper et al. 2009; Nissen-Meyer et al. 2010; Schneider and Sahl 2010).

#### 15.3.1 Lantibiotics

Among the very first bacteriocins identified and characterized was the lantibiotic nisin. It was first identified in the 1920s (Rogers 1928), but it was not until 1944 that its primary structure was determined (Mattick and Hirsch 1944). In 1988, the chemical synthesis of nisin was successfully achieved, resulting in confirmation of the proposed structure (Fukase et al. 1988). The biosynthesis of nisin was accomplished at the same time (Buchman et al. 1988). In recent years, nisin and its counterparts have received a lot of attention because of their possible applications and also because of the disclosure of its mode of action.
Lantibiotics are known to be posttranslationally modified and they received their name because lanthionine/methylanthione residues are constituents of their polypeptide chains. These modifications are made by dehydrating certain serine and/or threonine residues to didehydroalanine and didehydrobutyrine, respectively. Some of the modified residues are covalently linked to the sulfur of cysteines to form lanthionines and methyl-lanthionines in mature lantibiotics. A number of additional modifications can also be present in lantibiotics.

Back in the early 1990s, lantibiotics were divided into two subclasses: type A and type B (Jung 1991). The type A lantibiotics, which included nisin, galidermin, and epidermin, are elongated, amphiphilic, screw-shaped membrane depolarizing and permeabilizing cationic peptides up to 34 residues long and they act through the destruction of target bacteria membranes causing leakage of low molecular weight compounds that eventually leads to cell death. Structurally, the assemblage of (Me-)Lan bridges appears to very similar among the A-type lantibiotics. Type B lantibiotics include shorter (up to 19 residues) lantibiotics that have a globular structure, and some act by inhibiting enzyme activities that have impact on cell wall synthesis. Type B lantibiotics include mersacidin, actagardine, duramycins, and others. Since the number and diversity of lantibiotics have increased, many new lantibiotics did not fit into this simple classification and in 2005 van der Donk and co-workers divided the A group into two further subgroups (AI and AII) based on the modification system where the AI group is modified by two distinct enzymes (Lan B and C), whereas the lantibiotics modified by a single enzyme (LanM) are in group AII (Chatterjee et al. 2006). They also introduced a separate group comprising two-peptide lantibiotics (Chatterjee et al. 2006). At the same time, a revised classification scheme based on the sequence homology of prototypes of lantibiotics such as nisin and lacticin 481 was suggested by Cotter et al. (2005b). In 2009 a revised classification scheme was introduced (Piper et al. 2009), but the classification controversy has still not been settled.

The lacticin 482 group consists of at least 16 members and it has been reviewed previously (Dufour et al. 2007). This group of structural, homologous lantibiotics has become isolated from diverse bacteria, including Butyrivibrio isolates, Kocuria varians, Clostridium nexile, Butyrivibrio fibrisolvens, and LAB. The 481 group of lantibiotics are synthesized with a double-glycine leader and are secreted by a dedicated ABC transporter as originally shown to be the major secretion system among Class II bacteriocins (Havarstein et al. 1995). The double-glycine leaders and their dedicated ABC transporters have also been identified in gram-negative bacteria (Havarstein et al. 1994).

A list of the lantibiotics isolated from LAB is presented in Table 15.1. It is surprising to note that among the enterococci, the only lantibiotic found thus far is cytolysin, and this bacteriocin has been shown to be hemolytic.

### 15.3.1.1 Genes and Their Function

The biosynthesis machinery of lantibiotics is encoded by many genes located in operons. In addition, in many lantibiotics, the synthesis is under the control of a regulatory system that is encoded by additional genes. The biosynthetic and regulatory genes are organized in operons that may vary in how the genes are individually localized, but they are clustered on the genome and the genes involved are very much the same with only some minor differences. The self-protection mechanism of the producers can include more than one protection system, although in some lantibiotics such as nisin the immunity constitutes two mechanisms, an ABC-like transporter system that is encoded by three genes and/or by a single dedicated immunity gene (nisI). It should also be mentioned that the biosynthetic machinery of lacticin 481 (Xie et al. 2004) and nisin (Li et al. 2006) have been reconstituted in vitro with purified enzymes.
Table 15.1 Lantibiotics in LAB

<table>
<thead>
<tr>
<th>Lantibiotic</th>
<th>Producer</th>
<th>References</th>
<th>Features</th>
</tr>
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<tbody>
<tr>
<td>Nisin A</td>
<td>Lc. lactis</td>
<td>Gross and Morell (1971)</td>
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<tr>
<td>Nisin Z</td>
<td>Lc. lactis</td>
<td>de Vos et al. (1993)</td>
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<td>Nisin Q</td>
<td>Lc. lactis</td>
<td>Zendo et al. (2003)</td>
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<td>Nisin F</td>
<td>Lc. lactis</td>
<td>de Kwaadsteniet et al. (2008)</td>
<td></td>
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<tr>
<td>Nisin U</td>
<td>Str. uberis</td>
<td>Wirawan et al. (2006)</td>
<td></td>
</tr>
<tr>
<td>Nisin U2</td>
<td>Str. uberis</td>
<td>Wirawan et al. (2006)</td>
<td></td>
</tr>
<tr>
<td>Salivaricin X</td>
<td>Str. salivarius</td>
<td>Unpublished</td>
<td>Nisin-like</td>
</tr>
<tr>
<td>Lacticin 3147</td>
<td>Lc. lactis</td>
<td>Dougherty et al. (1998)</td>
<td>Two-peptide lantibiotic, d-ala residues</td>
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<tr>
<td>Lacticin J46</td>
<td>Lc. lactis</td>
<td>Huot et al. (1996)</td>
<td></td>
</tr>
<tr>
<td>Lacticin 481</td>
<td>Lc. lactis</td>
<td>Piard et al. (1993)</td>
<td>Prototype 481 subgroup</td>
</tr>
<tr>
<td>Salivaricin A</td>
<td>Str. salivarius</td>
<td>Wescombe et al. (2006)</td>
<td>481 subgroup</td>
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<tr>
<td>Salivaricin A2</td>
<td>Str. salivarius</td>
<td>Wescombe et al. (2006)</td>
<td>481 subgroup</td>
</tr>
<tr>
<td>Salivaricin A3</td>
<td>Str. salivarius</td>
<td>Wescombe et al. (2006)</td>
<td>481 subgroup</td>
</tr>
<tr>
<td>Salivaricin A4</td>
<td>Str. salivarius</td>
<td>Wescombe et al. (2006)</td>
<td>481 subgroup</td>
</tr>
<tr>
<td>Salivaricin A5</td>
<td>Str. salivarius</td>
<td>Wescombe et al. (2006)</td>
<td>481 subgroup</td>
</tr>
<tr>
<td>Salivaricin B</td>
<td>Str. salivarius</td>
<td>Hyink et al. (2007)</td>
<td>481 subgroup</td>
</tr>
<tr>
<td>Streptin</td>
<td>Str. pyogenes</td>
<td>Wescombe and Tagg (2003)</td>
<td>481 subgroup</td>
</tr>
<tr>
<td>Salivaricin A1</td>
<td>Str. pyogenes</td>
<td>Wescombe et al. (2006)</td>
<td>481 subgroup</td>
</tr>
<tr>
<td>Streprococcin A-FF22</td>
<td>Str. pyogenes</td>
<td>Tagg and Wannamaker (1976)</td>
<td>481 subgroup</td>
</tr>
<tr>
<td>BHT-Aa and Ab (Smb-like)</td>
<td>Str. ratus</td>
<td>Hyink et al. (2005)</td>
<td>Two peptides</td>
</tr>
<tr>
<td>Mutacin B-NY266</td>
<td>Str. mutans</td>
<td>Mota-Meira et al. (1997)</td>
<td></td>
</tr>
<tr>
<td>Mutacin 1140</td>
<td>Str. mutans</td>
<td>Hillman et al. (1998)</td>
<td></td>
</tr>
<tr>
<td>Mutacin I</td>
<td>Str. mutans</td>
<td>Tsang et al. (2005)</td>
<td></td>
</tr>
<tr>
<td>Mutacin K8</td>
<td>Str. mutans</td>
<td>Robson et al. (2007)</td>
<td></td>
</tr>
</tbody>
</table>

(continued)
15.3.1.2 Modification

The group A lantibiotics is divided into two groups based on the enzymatic mechanisms of modification (Pag and Sahl 2002; Chatterjee et al. 2006). Among class I lantibiotics, the dehydratase enzyme (LanB) converts Ser and Thr present in the precursor peptides to dehydroalanine (Dha) and dehydrobutyrine (Dhb), respectively. This reaction is followed by the addition of Cys thiols to Dha/Dhb to form the covalently thioether bond that is catalyzed by LanC cyclases. The final modified amino acid residues are lanthionine (Dha and Cys) and methyllanthionine (Dhb and Cysr). Class II lantibiotics are produced by bifunctional LanM-modifying enzymes, which are responsible for both dehydration and cyclization (Xie et al. 2004). The C-terminus of LanM proteins has sequence homology with the LanC enzymes, but LanM did not show any significant homology with the LanB enzymes (Siezen et al. 1996). The LanC-like domains in LanM proteins have been studied by structural analysis and mutagenesis, which have provided insights into the cyclization steps in lantibiotic biosynthesis (Okeley et al. 2003; Li et al. 2006; Li and van der Donk 2007). The dehydration reaction by the synthetase is still not understood.

Ribosomally synthesized polypeptides only contain the l-isoform of amino acids, and it was a surprise when it was shown that the lantibiotic lactocin S contains three d-alanine residues (Skaugen et al. 1994). These were the first d-amino acid residues in ribosomally synthesized bacterial proteins to be found. Later, it was also shown that the two-peptide lantibiotic lacticin 3147 also has such d-alanine residues (Ryan et al. 1999). The enzyme involved in this l-to-d post-translational conversion was later identified in the lacticin 3147 system. It was reported that the

<table>
<thead>
<tr>
<th><strong>Lantibiotic</strong></th>
<th><strong>Producer</strong></th>
<th><strong>References</strong></th>
<th><strong>Features</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Mutacin II</td>
<td><em>Str. mutans</em></td>
<td>Woodruff et al. (1998)</td>
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<tr>
<td>SmbAB</td>
<td><em>Str. mutans</em></td>
<td>Yonezawa and Kuramitsu (2005); Petersen et al. (2006)</td>
<td></td>
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<tr>
<td>Bovicin HJ50</td>
<td><em>Str. bovis</em></td>
<td>Xiao et al. (2004)</td>
<td></td>
</tr>
<tr>
<td>Bovicin HC5</td>
<td><em>Str. bovis</em></td>
<td>Mantovani et al. (2002)</td>
<td></td>
</tr>
<tr>
<td>Macedocin</td>
<td><em>Str. macedonicus</em></td>
<td>Georgalaki et al. (2002)</td>
<td></td>
</tr>
<tr>
<td>Plantaricin W</td>
<td><em>Lb. plantarum</em></td>
<td>Holo et al. (2001)</td>
<td>Two-peptide lantibiotic</td>
</tr>
<tr>
<td>Lactocin S</td>
<td><em>Lb. sakei</em></td>
<td>Mortvedt et al. (1991)</td>
<td>d-alanine residues</td>
</tr>
<tr>
<td>Cytolysin</td>
<td><em>E. faecalis</em></td>
<td>Booth et al. (1996)</td>
<td>Two-peptide lantibiotic; also hemolytic</td>
</tr>
</tbody>
</table>
The putative enzyme LtnJ bears a significant similarity to dehydrogenases–reductases, particularly zinc-containing alcohol dehydrogenases and NAD(P)H-dependent quinone oxidoreductases of the zinc-containing alcohol dehydrogenase superfamily (Cotter et al. 2005c). Deletion of this enzyme (LtnJ) led to the participating residues remaining as Dha intermediates, and caused a dramatic reduction in the antimicrobial activity of the producer. The chirality of the three d-alanines present in lacticin 3147 is crucial for antimicrobial activity, which was confirmed when the three amino acid residues were substituted by l-alanines.

15.3.1.3 Lantibiotic Immunity

The producer of a bacteriocin has to protect itself against its own bacteriocin, and such self-protection is referred to as immunity. Dedicated immunity systems have been identified for lantibiotics, but except for the immunity of nisin, little is known how immunity works.

In nisin and its counterparts, the immunity of the producers can constitute two mechanisms: an ABC-like transporter system, which is encoded by three genes \textit{nisEFG}, and a single dedicated immunity gene \textit{(nisI)}. The \textit{nisEF} are the two membrane-embedded components, and \textit{nisG} encodes the cytoplasmatic entity of the ABC transporter. The putative immunity protein NisI is located on the outer surface of the cytoplasmic membrane and has been shown to be a lipoprotein (Qiao et al. 1995). Functional studies have shown that both immunity systems are needed to achieve maximum protection (Ra et al. 1999). Similar immunity systems have been shown for other lantibiotics, including the two-peptide lantibiotics (McAuliffe et al. 2000; Draper et al. 2009), but a detailed insight of how the immunity activity of LAB lantibiotics work has yet to be determined.

There is experimental evidence to show that the C-terminal domain of NisI is involved in the binding of nisin (Koponen et al. 2004) and that this interaction involves NisEFG (Takala and Saris 2006).

A recent paper showed that the E loop of the NukF protein, which is part of the ABC transporter immunity function of nukacin-IAK-1, is of crucial important for the immunity function (Okuda et al. 2010). In addition to the dedicated immunity system, a number of genes including the immune mimicry of immunity homologue genes have been shown to decrease the susceptibility to lantibiotics (Draper et al. 2009; Collins et al. 2010a; Collins et al. 2010b). The presence of such genes may partly explain why the inhibitory concentration of lantibiotics varies between and within bacterial species.

15.3.1.4 Diversity, Structure, and Mode of Action

This section refers to a recent, detailed, published review that deals with the diversity of the structural features and biological activity of lantibiotics (Asaduzzaman and Sonomoto 2009). Lantibiotics are found within different genera and species of gram-positive bacteria and many lantibiotic variants must have been disseminated via various ways of horizontal gene transfer and slowly developed into new variants. This is particularly demonstrated by nisin, where numerous nisin-like lantibiotics have been isolated from different bacteria (Table 15.1). The structure of several LAB lantibiotics is known and most attention has been paid to nisin, lacticin 481, and lacticin 3147.

Lacticin 481, which is a bacteriocin produced by \textit{Lactococcus lactis} ssp. \textit{lactis}, contains 27 amino acid residues and the posttranslatory modified residues dehydrobutyrine and the thioether-bridging lanthionine and 3-methyllanthionine. Lacticin 481 belongs to a structurally distinct group of lantibiotics.
that also comprises streptococcin A-FF22 and various salivaricins (Table 15.1). Multidimensional nuclear magnetic resonance (NMR) spectroscopy combined with chemical modifications and peptide sequences identified the lanthionine bridge pattern in and the structure of both peptides (A1 and A2) of lacticin 3147 (Martin et al. 2004). NMR spectroscopy was also used for conformational analysis of both peptides. It was shown that the A1 peptide structure adopts the globular mercacidin type B structure, whereas the A2 peptide adopts an α-helical elongated structure, probably similar to lactocin S, another modified d-alanine, one-peptide lantibiotic. The molar ratio of two peptides for optimal antimicrobial activity is 1:1, and it has been suggested that the role of peptide A1 is lipid II target recognition while peptide A2 is the pore-forming part of lacticin 3147 (Martin et al. 2004). It has also been demonstrated that sequential activity of the two peptides is needed. The A1 peptide has to be bound to the target before the action of the A2 peptide, which leads to the depolarization of the membrane of the target bacteria (Martin et al. 2004). When the two peptides are combined, the killing of target bacteria takes place at nanomolar concentrations. It should also been mentioned that structural studies have also been performed on a number of lantibiotics from non-LAB and these structures have also elucidated structural features of the LAB (Aymerich et al. 1996; O’Connor et al. 2007; Shenkarev et al. 2010; Abriouel et al. 2011).

15.3.2 Nonlantibiotics (Class II)

Class II bacteriocins constitute a large and diverse group of antimicrobial peptides that are divided into several different subgroups. As with Class I, Class II also comprises small heat-stable, cationic, and hydrophobic/amphiphilic peptides.

In this review we divide Class II bacteriocins into the following subclasses: IIa, the pediocin-like bacteriocins; IIb, the two-peptide bacteriocins; IIc, the bacteriocins that do not fit into this grouping; IId, the leaderless bacteriocins; and IIe, the circular bacteriocins.

15.3.2.1 IIa Bacteriocins

This is the largest subclass (Table 15.2). They are a very well-defined group of peptides, both by their structure as well as by their mode of action and target specificities. Numerous reviews have already covered these peptides and some recent reviews specifically focused on them (Fimland et al. 2005; Drider et al. 2006; Nissen-Meyer et al. 2009; Papagianni and Anastasiadou 2009). These bacteriocins are between 36 (plantaricin C19) and 49 (ubericin A) amino acid residues long.

15.3.2.1.1 Structure and Diversity

On the basis of the amino acid sequence of 28 Class IIa bacteriocins, two functional regions have been identified: (i) a highly conserved positive and hydrophilic N-terminal region (up to 17 amino acid residues long) and (ii) a C-terminal less well conserved hydrophobic region. It was noticed early on that the highly conserved N-terminal region with its Cys–Cys bridge is needed for the antimicrobial activity (Eijsink et al. 1998). The 100% conserved sequence in the N-terminal domain is YGNGVXXXXXXCXV. It has also been suggested that Class IIa should be divided into three or four different groups on the basis of sequence similarities and differences in the more variable C-terminal domain (Johnsen et al. 2005b). This subgrouping is based on length, tryptophan residues, or cysteine bridges present in the C-terminal part of the peptides, as well as on structural analysis (Table 15.2). The leader sequence has been characterized for 27 of the Class IIa bacteriocins, and they all carry a leader sequence that keeps the pre-bacteriocin from being
<table>
<thead>
<tr>
<th>Subgroup 1</th>
<th>Bacteriocin</th>
<th>Producer</th>
<th>Mature Sequence</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subgroup 1</td>
<td>Enterocin A</td>
<td><em>E. faecium</em></td>
<td>TTHSGKYYGNGVYCTKNKCTVDWAKATTCIAGMSIGGFLGGAIPG--KC</td>
<td>Aymerich et al. (1996)</td>
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<td></td>
<td>Divercin V41</td>
<td><em>Ca. divergens</em></td>
<td>TKYYGNGVYCNSKKCWVDWGQASGCIGQTVVGGWLGGAIPG--KC</td>
<td>Metivier et al. (1998)</td>
</tr>
<tr>
<td></td>
<td>Divergicin M35</td>
<td><em>Ca. divergens</em></td>
<td>TKYYGNGVYCNSKKCWVDWGTAQGCID--VVIGQLGGGIPGKGKC</td>
<td>Tahiri et al. (2004)</td>
</tr>
<tr>
<td></td>
<td>Bavaricin MN</td>
<td><em>Lb. sakei</em></td>
<td>TKYYGNGVYCNSKKCWVDWGQAAGGIGQTVVXGWLGGAIPG--KC</td>
<td>Kaiser et al. (1996)</td>
</tr>
<tr>
<td></td>
<td>Coagulin</td>
<td><em>B. cogulans</em></td>
<td>KYYGNGVTCGKHSCSVDWGKATTCIINNGAMAWATGGHQGTHKC</td>
<td>Le Marrec et al. (2000)</td>
</tr>
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<td></td>
<td>Pediocin PA-1</td>
<td><em>P. acidilactici</em></td>
<td>KYYGNGVTCGKHSCSVDWGKATTCIINNGAMAWATGGHQGTHKC</td>
<td>Henderson et al. (1992); Nieto Lozano et al. (1992)</td>
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<td></td>
<td>Mundticin</td>
<td><em>E. mundtii</em></td>
<td>KYYGNGVSCNKKGCSVDWGKAIGIIGNNNAAANLTGGAAGWSK</td>
<td>Bennik et al. (1998)</td>
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<td>Piscicocin CS526</td>
<td><em>Ca. piscicola</em></td>
<td>KYYGNGLSXNKKGXTVDWGTAIGIIGNNAAANLTTGGAAGxNK</td>
<td>Yamazaki et al. (2005)</td>
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<td>Piscicocin 126/V1a</td>
<td><em>Ca. piscicola</em></td>
<td>KYYGNGVSCNKNGCTVDWSKAIGIIGNNAAANLTTGGAAGWNKG</td>
<td>Bhugaloo-Vial et al. (1996); Jack et al. (1996)</td>
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<td>Sakacin P</td>
<td><em>Lb. sakei</em></td>
<td>KYYGNGVHCGKHSCTVDWGTAIGNIGNNAAANWATGGNAGWNK</td>
<td>Tichaczek et al. (1994)</td>
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<td></td>
<td>Leucocin C</td>
<td><em>Leu. mesenteroides</em></td>
<td>KYYGNGVSCNKSGCSVDWSKAISIIGNNAVANLTTGGAAGWKS</td>
<td>Fimland et al. (2002b)</td>
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<td></td>
<td>Listeriocin 743A</td>
<td><em>Li. innocua</em></td>
<td>KYYGNGVSCNKSGCSVDWSKAISIIGNNAVANLTTGGAAGWKS</td>
<td>Kalmokoff et al. (2001)</td>
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<td>Sakacin 5X</td>
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<td>Enterocin CRL35/</td>
<td><em>E. mundtii</em></td>
<td>KYYGNGVSCNKSGCSVDWSKAISIIGNNAVANLTTGGAAGWKS</td>
<td>Farias et al. (1996); Kawamoto et al. (2002)</td>
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<td>Mundticin KS</td>
<td><em>E. mundtii</em></td>
<td>KYYGNGVSCNKSGCSVDWSKAISIIGNNAVANLTTGGAAGWKS</td>
<td>(continued)</td>
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### Table 15.2 Class IIa Bacteriocins and Their Subgroups (Continued)

<table>
<thead>
<tr>
<th>Bacteriocin</th>
<th>Producer</th>
<th>Mature Sequence</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Subgroup 1</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Avicin A</td>
<td>E. avium</td>
<td>KYYGNGVSCNKKGCSVDWGKAISIIGNNSAANLATGGAAGWKS</td>
<td>Birri et al. (2010)</td>
</tr>
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<td>Mundticin L</td>
<td>E. mundtii</td>
<td>KYYGNGVSCNKKGCSVDWGKAIGIIGNNSAANLATGGAAGWKS</td>
<td>Feng et al. (2009)</td>
</tr>
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<td>Enterocin HF</td>
<td>E. faecium</td>
<td>KYYGNGVSCNKKGCSVDWGKAIGIIGNNSAANLATGGAAGWKS</td>
<td>Almeida et al. (2009)</td>
</tr>
<tr>
<td>Bavaricin A</td>
<td>Lb. bavaricus</td>
<td>KYYGNGVHXGKHSXTVDWGTAIGNIGNNAAANXATGXNAGG</td>
<td>Larsen et al. (1993)</td>
</tr>
<tr>
<td>Ubericin A</td>
<td>Str. uberis</td>
<td>KTVNYGNGLYCNQKKCWVNWSETATTIVNNSIMNGLTGGNAGWHSGGRA</td>
<td>Heng et al. (2007)</td>
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<tr>
<td><strong>Subgroup 2</strong></td>
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<td></td>
</tr>
<tr>
<td>Leucocin A</td>
<td>Leuconostoc spp.</td>
<td>KYYGNGVHCTKSGCSVNWGEAFSAGVHRLANGGNGFW</td>
<td>Hastings et al. (1991)</td>
</tr>
<tr>
<td>Mesentericin Y105</td>
<td>Leu. mesenteroides</td>
<td>KYYGNGVHCTKSGCSVNWGEAASAGIHRLANGGNGFW</td>
<td>Hechard et al. (1992)</td>
</tr>
<tr>
<td>Sakacin G</td>
<td>Lb. sakei</td>
<td>KYYGNGVSCNQKKCWVNWGQATAGHGKX</td>
<td>Simon et al. (2002)</td>
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<td>Plantaricin 423</td>
<td>Lb. plantarum</td>
<td>KYYGNGVSCNQKKCWVNWGQATAGHGKX</td>
<td>Van Reenen et al. (2003)</td>
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<td>Plantaricin C19</td>
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<td>KYYGNGVSCNQKKCWVNWGQATAGHGKX</td>
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<td><strong>Subgroup 3</strong></td>
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<td>Curvacin A/Sakacin A</td>
<td>Lb. curvatus/Lb. sakei</td>
<td>KHYGNGVYCNKSKCWLWGAQAGG</td>
<td>Holck et al. (1992); Tichaczek et al. (1992)</td>
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<tr>
<td>Carnobacteriocin BM1</td>
<td>Ca. piscicola</td>
<td>KHYGNGVYCNKSKCWLWGAQAGG</td>
<td>Quadri et al. (1994)</td>
</tr>
<tr>
<td>Enterocin P</td>
<td>E. faecium</td>
<td>KHYGNGVYCNKSKCWLWGAQAGG</td>
<td>Cintas et al. (1997)</td>
</tr>
<tr>
<td>Piscicoin V1b</td>
<td>Ca. piscicola</td>
<td>KHYGNGVYCNKSKCWLWGAQAGG</td>
<td>Bhugaloo-Vial et al. (1996)</td>
</tr>
</tbody>
</table>

(continued)
toxic, but it is removed during secretion. The double glycine leader sequence is found in 22 Class IIa bacteriocins, while only six have a sec-dependent leader.

Several structures of Class IIa bacteriocins have been determined (Uteng et al. 2003; Sprules et al. 2004a; Haugen et al. 2005), and the structural elements common to these bacteriocins are an N-terminal β-sheet domain with its Cys–Cys bridge hinged to an α-helical structure ending up with a hairpin domain in the C-terminus and a flexible C-terminal end (Figure 15.1). It is also of great interest to observe how this type of bacteriocin is disseminated among numerous genera of LAB, but it has not been found in lactococci.

### 15.3.2.1.2 Immunity

Like all bacteriocins, the pediocin-like bacteriocins also have a dedicated immunity protein that protects the producers from being killed. Such immunity proteins vary between 88 and 118 amino acid residues, but there is significantly more variation in their primary structure than is

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**Table 15.2 Class IIa Bacteriocins and Their Subgroups (Continued)**

<table>
<thead>
<tr>
<th>Bacteriocin</th>
<th>Producer</th>
<th>Mature Sequence</th>
<th>References</th>
</tr>
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<td><em>P. pentosaceus</em></td>
<td>ATYDGCSCVRMAVQQQYKAKRMGKQKIVNGWTQHGPWAHR</td>
<td>Diep et al. (2006)</td>
</tr>
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<td>Bacteriocin 31</td>
<td><em>E. faecalis</em></td>
<td>ATQRGCSCVRMAVQQQYKAKRMGKQKIVNGWTQHGPWAHR</td>
<td>Tomita et al. (1996)</td>
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<tr>
<td>Bacteriocin RC714</td>
<td><em>E. faecium</em></td>
<td>ATQRGCSCVRMAVQQQYKAKRMGKQKIVNGWTQHGPWAHR</td>
<td>del Campo et al. (2001)</td>
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<td>Hiracin JM79/Bacteriocin T8</td>
<td><em>E. hirae/E. faecium</em></td>
<td>ATQRGCSCVRMAVQQQYKAKRMGKQKIVNGWTQHGPWAHR</td>
<td>de Kwaadsteniet et al. (2006); Sanchez et al. (2007)</td>
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<td><em>E. faecalis</em></td>
<td>ATQRGCSCVRMAVQQQYKAKRMGKQKIVNGWTQHGPWAHR</td>
<td>Eguchi et al. (2001)</td>
</tr>
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<td>Carnobacteriocin B2</td>
<td><em>Ca. piscicola</em></td>
<td>ATQRGCSCVRMAVQQQYKAKRMGKQKIVNGWTQHGPWAHR</td>
<td>Quadri et al. (1994)</td>
</tr>
<tr>
<td>Lactococcin MMFII</td>
<td><em>Lc. lactis</em></td>
<td>ATQRGCSCVRMAVQQQYKAKRMGKQKIVNGWTQHGPWAHR</td>
<td>Ferchichi et al. (2001)</td>
</tr>
<tr>
<td>Bacteriocin MC4-1</td>
<td><em>E. faecalis</em></td>
<td>ATQRGCSCVRMAVQQQYKAKRMGKQKIVNGWTQHGPWAHR</td>
<td>Flannagan et al. (2008)</td>
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<tr>
<td>SRCAM 1580</td>
<td><em>B. circulans</em></td>
<td>ATQRGCSCVRMAVQQQYKAKRMGKQKIVNGWTQHGPWAHR</td>
<td>Svetoch et al. (2005)</td>
</tr>
<tr>
<td><strong>CONSENSUS</strong></td>
<td></td>
<td>YGNGV C C V W A</td>
<td></td>
</tr>
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</table>
observed among their dedicated bacteriocins (Drider et al. 2006). By pairwise alignment, 3–100% sequence homology was found; the most distant ones are the immunities of carnobacteriocin B2 and plantaricin 423, whereas the immunity proteins of pediocin PA-1 and coagulin are identical. A phylogenetic analysis of the immunity proteins and their bacteriocins can be seen in Figure 15.2. The different subgroups of the Class IIa bacteriocins are as indicated (see text of Figure 15.2). Numerous investigations have been performed on these proteins, including studies on specificity (cross-immunity) and structural functional analyses, including their role in the mechanisms protecting bacteriocin producers (Quadri et al. 1995; Fimland et al. 2002a; Dalhus et al. 2003; Figure 15.1 Structure of Class IIa bacteriocin leucocin A. Superimposition of ribbon structure is based on the study of Fregeau Gallagher and co-workers (Fregeau Gallagher, Sailer et al. 1997).

Figure 15.2 Phylogram: Class IIa bacteriocins and their cognate immunity protein, subgroups as indicated. Phylogenetic tree was constructed using phylogeny.fr interface (Dereeper et al. 2008): Protein sequences were aligned using MUSCLE (Edgar 2004), and tree was constructed using PhyML (Guindon and Gascuel 2003) and visualized by TreeDyn (Chevenet et al. 2006).
Johnsen et al. 2004; Sprules et al. 2004b; Diep et al. 2007; Martin-Visscher et al. 2008a). It is surprising to observe the specificity of immunity, and cross-immunity is hardly seen between the various Class IIa systems.

The chemical structure of several of the Class IIa immunity proteins has been resolved. NMR spectroscopy or X-ray crystallography have been performed on five immunity proteins: enterocin A (EntA-Im) (Johnsen et al. 2005a), piscicolin 126 (Pisl), carnobacteriocin B2 (ImB2), sakacin P, and curvacin A (Sprules et al. 2004b; Haugen et al. 2005; Johnsen et al. 2005b; Martin-Visscher et al. 2008a). In aqueous solution, they are organized into a globular antiparallel four-helix bundle structure.

Compared with ImB2 and EntA-Im, the PisI immunity protein has a substantially longer and more flexible N-terminus, but a shorter C-terminus. No direct interaction between the bacteriocin and the immunity protein has been observed by NMR, neither in aqueous solution nor in membrane-mimicking solvents. This was also found for the lactococcin A immunity protein, a system quite different from the pediocins. However, it has shown that such immunity proteins actually bind strongly in a complex with the bacteriocins and the receptor, forming a tight tri-complex that prevents the bacteriocin from killing the target cell (Diep et al. 2007). It has also been observed that the C-terminal part of the bacteriocins and the C-terminal part of their dedicated immunity proteins are involved in their specific and probably indirect interaction (Nissen-Meyer et al. 2009).

15.3.2.2 Two-Peptide Bacteriocins

The unique feature of these bacteriocins is that two peptides are needed to express antimicrobial activity or to have optimal antimicrobial activity. The optimal activity is usually obtained when the two peptides act together in a molar ratio of 1:1. To define a two-peptide bacteriocin, only one immunity protein is required to protect the producer from committing suicide. All two-peptide bacteriocins identified thus far are synthesized with a double-glycine leader and secreted by the dedicated ABC transporter with the typical N-terminal proteolytic domain (Havarstein et al. 1995). One could speculate as to whether the two-peptide bacteriocins are a result of the fusion of two single bacteriocins and whether the two individual immunity genes have been replaced by one.

15.3.2.2.1 Immunity Proteins

The immunity proteins of the two-peptide bacteriocins are structurally different from the Class IIa proteins. They vary in size from the smallest, thermophilin 12, being only 52 amino acid residues long to the immunity of plantaricin EF, being 257 residues long. Structural in silico analysis predicts that they could contain from two up to seven transmembrane helices and that the proteins are membrane embedded. NMR spectroscopic or X-ray crystallographic analysis have been performed on several Class IIb bacteriocins and confirmed that these proteins are structured into a bundle of four helices (Sprules et al. 2004b; Johnsen et al. 2005b; Kim et al. 2007; Martin-Visscher et al. 2008a; Jeon et al. 2009). In a recent work, we reported that several bacteriocin loci encode proteins belonging to the Pfam Abi protein family (Pfam accession no. PF02517) (Kjos et al. 2010b). These loci also include the two-peptide bacteriocins EF and JK in the plantaricin (pbl) locus of Lb. plantarum (Diep et al. 1996; Anderssen et al. 1998), as well as in the multibacteriocin pnc locus of Streptococcus pneumoniae (Lux et al. 2007), and the streptolysin S (sag) locus found in group A streptococci (Nizet et al. 2000). The Abi protein family consists of putative membrane-bound metalloproteases. While they are involved in membrane anchoring of proteins in eukaryotes, little
is known about their function in prokaryotes (Pei and Grishin 2001). Some of the Abi-like proteins (PlnI in \textit{Lb. plantarum}, PncO in \textit{Str. pneumoniae}, and SagE in \textit{Str. pyogenes}) were found to be bacteriocin self-immunity proteins in experimental results from gene knockout studies (Datta et al. 2005; Lux et al. 2007), also indicated by their gene localization (i.e., being associated next to bacteriocin structural genes). Other bacteriocin-associated Abi-like proteins (e.g., PlnP and PlnTUVW in \textit{Lb. plantarum} and PncP in \textit{Str. pneumoniae}) do not seem to be immunity proteins but possess completely unknown functions.

15.3.2.3 Diversity, Structure, and Mode of Action

At least 17 two-peptide bacteriocins have been identified (van Belkum et al. 1991a; Nissen-Meyer et al. 1992; Allison et al. 1994; Jimenez-Diaz et al. 1995; Diep et al. 1996; Marciset et al. 1997; Anderssen et al. 1998; McCormick et al. 1998; Stephens et al. 1998; Cuozzo et al. 2000; Qi et al. 2001; Flynn et al. 2002; Franz et al. 2002; Maldonado et al. 2002; Maldonado et al. 2003; Vaughan et al. 2003; Zendo et al. 2006; Barrett et al. 2007; Maldonado-Barragán et al. 2009; Hu et al. 2010); some of the individual peptides have been reported to have a little antimicrobial activity, but when both peptides are combined a strong synergistic activity is seen. Nevertheless, in most cases when purified/synthesized peptides have been studied, both peptides absolutely seem to be needed. Several excellent review articles have dealt with the two-peptide bacteriocins (Nissen-Meyer and Sletten 1991; Garneau et al. 2002; Oppegard et al. 2007).

Numerous studies related to the structure and modes of action have been performed. The early mode of action studies showed that, like many other bacteriocins, the target of the two peptide-bacteriocins was the membrane, where they caused leakage (Moll et al. 1998; Moll et al. 1999a) of monovalent cations, depletion of the ATP pool, dissipation of the membrane potential, and eventually death of the susceptible target organism. It has also been shown that the individual peptides bind irreversibly to target bacteria and cannot migrate to another cell when bound, which suggests that the peptides bind independently of each other, though probably nonspecifically since no antimicrobial activity was seen then (Moll et al. 1998).

Of the two-peptide bacteriocins, lactococcin G is the most studied when it comes to the mode of action. It is believed that it requires a target/receptor but none have been identified yet. As already mentioned, both peptides adapt to amphiphilic helical structures upon exposure to membrane-mimicking solvents. Furthermore, when the two peptides are together, they interact, somehow manifested by additional structuring, as seen by circular dichroism (CD) analysis. By use of NMR spectroscopy, it has been shown that lactococcin G (Rogne et al. 2008), plantaricin EF (PlnP/E/F) (Fimland et al. 2008), and plantaricin JK (PlnJ/K) (Rogne et al. 2009) are adapted into α-helical structures, while the C- and N-terminal parts of the peptides are less well shaped. The structural studies performed with the two-peptide bacteriocins PlnE/F and PlnJ/K under various conditions by CD showed that while the peptides were unstructured under aqueous conditions, they adopted a partly α-helical structure in the presence of trifluoroethanol, micelles of dodecylphosphocholine, and negatively charged dioleoylphosphatidylglycerol (DOPG) liposomes (Hauge et al. 1999). Enhanced structuring was observed when both peptides (PlnE and PlnF or PlnJ and PlnK) were added simultaneously to DOPG liposomes. Preliminary CD studies showed that both peptides of brochocin C apparently adopt β-sheet structures (Garneau et al. 2003); however, further investigations are needed to reveal the complete structure of this bacteriocin and their like.

Structural studies of lactococcin G were followed up by a mutation analysis of the helical regions of the peptides (Oppegard et al. 2008). This study focused on tryptophan and tyrosine residues that are preferentially found in the membrane interphase in membrane-associated protein
(Killian and von Heijne 2000) and on the putative helix–helix motif GXXXG found both in α peptides (G_{7–G_{11}} and G_{18–G_{22}}) and β peptides (G_{18–G_{22}}). This study supports the notion that the helical motifs of the two peptides interact in a dimeric helical manner, penetrating the membrane flanked by tryptophan residues in the membrane interphase. The model also suggests that the N-termini of the peptides are directed outward when they are embedded in the membrane. How this model is consistent with a receptor model is too early to speculate about since the receptor, if any, has not been identified.

15.3.2.4 Bacteriocins without an N-Terminal Leader Sequence

A few leaderless bacteriocins have been identified in LAB (Cintas et al. 1998). Such antimicrobial peptides have been known for some time in non-LAB, such as staphylococci and propionic acid bacteria (Watson et al. 1988; Donvito et al. 1997; Faye et al. 2011), but such bacteriocins have since also been isolated from both *E. faecium* and *E. faecalis* (Cintas et al. 1998; Cintas et al. 2000; Sanchez-Hidalgo et al. 2003; Yamamoto et al. 2003; Martin-Platero et al. 2006). Curiously, several bacteriocins consecutively encode *orf* (up to four *orf*) with significant sequence identity, and it has been shown that they act synergistically (Cintas et al. 1998; Netz et al. 2001). The enterocins do not possess hemolytic activity, as seen with some of the *Staphylococcus* bacteriocins/hemolytic peptides (Watson et al. 1988; Donvito et al. 1997; Cintas et al. 1998). The enterocin L50A (EntL50A, 44 amino acid residues) and enterocin L50B (EntL50B, 43 amino acid residues) have 72% sequence identity. Both bacteriocins possess antimicrobial activity on their own, with EntL50A being the most active; however, when the two bacteriocins were combined, a considerable synergism was observed that varied between indicator strains (Cintas et al. 1998; Cintas et al. 2000). Presently, we do not know much about their mechanism of action but some results suggest that an ABC transporter both externalizes the peptides as well as protects the producer against the lethal activity of the peptides.

15.3.2.5 Cyclic Bacteriocins

Such bacteriocins refer to the ribosomally synthesized antimicrobial activity of which the N-terminal and C-terminal ends are covalently linked by an amide bond. The circular peptide structure encloses 58–70 amino acid residues, which are cationic and contain a large number of hydrophobic residues (Maqueda et al. 2008). They show significant stability over a wide range of temperature and pH levels and they are unaffected by a variety of proteases but not endoproteases (Galvez et al. 1986). The antimicrobial activities of several circular bacteriocins are fairly broad and are also directed against pathogens that include staphylococci, enterococci, listeria, and clostridia. For many years, AS-48 (Galvez et al. 1989; Samyn et al. 1994) was the only circular bacteriocin known, but several such bacteriocins have now been isolated and characterized (see Table 15.3).

15.3.2.5.1 Genetics

The genetic determinants of AS-48 were identified in 2003 (Martinez-Bueno et al. 1998). A region of 7.8 kb was defined in the conjugative plasmid pMB2 as encoding for the complete production of the AS-48 bacteriocin. The following six genes were initially identified: the structural gene of AS-48 followed by *as-48B, as-48C, as-48C_1, as-48D,* and *as-48D*. Further analysis revealed an additional four genes, *as-48EFGH*, necessary for full AS-48 expression (Diaz et al. 2003). The *as-48EFGH* operon encoded an ABC transporter that is apparently involved in the bacteriocin
immunity of the producer (Diaz et al. 2003). As most often observed, the genes involved in bacteriocin synthesis are clustered, and this is also the case for cyclic bacteriocins where the genes needed are organized into two operons that are coordinatively expressed. The genetic constituents needed for the biosynthesis of other circular bacteriocins have been cloned and sequenced (Kawai et al. 2004a). A detailed analysis of the genetic elements (an operon structure with five genes) encoding uberolysin production in \textit{Str. uberis} strain 42 has also been published (Wirawan et al. 2007). Bioinformatic analysis has identified genes with putative roles in its biosynthesis, including bacteriocin processing, immunity, and secretion. From this analysis, it seems likely that the various cyclic bacteriocins are dependent on the same machinery for their biosynthesis, but with some modifications (Maqueda et al. 2008).

<table>
<thead>
<tr>
<th>Bacteriocin</th>
<th>Producing Species</th>
<th>Leader Sequence</th>
<th>Amino Acid Residues</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>AS48</td>
<td>\textit{Enterococcus feacalis}</td>
<td>35</td>
<td>70</td>
<td>Galvez et al. (1986); Martínez-Bueno et al. (1994); Maqueda et al. (2004); Maqueda et al. (2008)</td>
</tr>
<tr>
<td>Gassericin A</td>
<td>\textit{Lactobacillus gasseri}</td>
<td>33</td>
<td>58</td>
<td>Kawai et al. (1998); Kawai et al. (2004a)</td>
</tr>
<tr>
<td>Reutericin 6</td>
<td>\textit{Lactobacillus reuteri}</td>
<td>33</td>
<td>58</td>
<td>Kabuki et al. (1997); Kawai et al. (2004a)</td>
</tr>
<tr>
<td>Acidocin B</td>
<td>\textit{Lactobacillus acidophilus}</td>
<td>33</td>
<td>58</td>
<td>Leer et al. (1995)</td>
</tr>
<tr>
<td>Butyrivibriocin</td>
<td>\textit{Butyrivibrio fibrisolvens}</td>
<td>22</td>
<td>78</td>
<td>Kalmokoff and Teather (1997); Kalmokoff et al. (2003)</td>
</tr>
<tr>
<td>Uberolycin</td>
<td>\textit{Streptococcus uberis}</td>
<td>3</td>
<td>78</td>
<td>Wirawan et al. (2007)</td>
</tr>
<tr>
<td>Circularin A</td>
<td>\textit{Clostridium bejerinckii}</td>
<td>6</td>
<td>69</td>
<td>Kemperman et al. (2003)</td>
</tr>
<tr>
<td>Garvicin ML</td>
<td>\textit{Lactococcus garvieae}</td>
<td>3</td>
<td>60</td>
<td>Borrero et al. (2011)</td>
</tr>
<tr>
<td>Lactocyclicin Q</td>
<td>\textit{Lactococcus sp.}</td>
<td>2</td>
<td>61</td>
<td>Sawa et al. (2009)</td>
</tr>
<tr>
<td>Subtilosin A and A1</td>
<td>\textit{Bacillus subtilis}</td>
<td>8</td>
<td>35</td>
<td>Kawulkka et al. (2004); Huang et al. (2009)</td>
</tr>
<tr>
<td>Carnocyclin A</td>
<td>\textit{Carnobacterium maltaromaticum}</td>
<td>4</td>
<td>60</td>
<td>(Martin-Visscher et al. (2008b); Martin-Visscher et al. (2009)</td>
</tr>
</tbody>
</table>
15.3.2.5.2 Structure–Function–Mode of Action

At present, 10 cyclic bacteriocins have been identified (Table 15.3). Among these, it has been shown that the primary amino acid sequences of gassericin A and reutericin 6 are identical, except for the presence of two d-alanines in gassericin A and one d-alanine in reutericin 6. These differences are also reflected in different spectra of activity and secondary structure profiles (Kawai et al. 2004b). Acidocin B is considered as a putative circular protein, as it shows 98% sequence identity to gassericin A and reutericin 6, but its circular nature has not been confirmed (Leer et al. 1995). Subtilosin A represents an atypical group of cyclic bacteriocins because it contains unique thioether bridges that covalently link cysteine sulfurs to the \( \alpha \)-carbon of other residues, being considerably shorter than other cyclic bacteriocins, as well as being anionic (Kawulka et al. 2004; Maqueda et al. 2008). In this context, it is relevant to mention the bacteriocin thuricin CD that consists of two distinct peptides and kills a wide range of *Clostridium difficile* isolates. Thuricin CD also contains the same modification of the carbon peptide backbone as seen in subtilosin A (Rea et al. 2010). These two bacteriocins will not be included further in this review.

The cationic circular AS-48 peptide (70 amino acid residues and an isoelectric point close to 10.5) is by far the most studied cyclic bacteriocin and it has been reported to exert both bactericidal and bacteriostatic activity, but important details of its mode of activity are still missing. The work of Galvez et al. (1991) showed that AS-48 induced ion permeation, which was accompanied by the collapse of the cytoplasmic membrane potential. Active transport by cytoplasmic membrane vesicles is also impaired by AS-48. At low concentrations, this peptide also permeabilizes liposomes, causing free migration of low molecular weight compounds without the requirement for a membrane potential. Higher SAS-48 concentrations induce severe membrane disorganization, as observed by electron microscopy, such as of aggregates and multilamellar structures. Electrical measurements suggest that AS-48 can form channels in lipid bilayers (Galvez et al. 1991).

A number of biological effects have been recorded when *L. monocytogenes* is exposed to AS-48 (Mendoza et al. 1999). The cell wall and the membrane structure were heavily affected by AS-48 and ghost cells were observed. *L. monocytogenes* can adapt to enterocin AS-48, where the cell wall thickness increases (Sanchez-Barrena et al. 2003) and the lipid composition changes (300). Resistant cells were less affected by muramidases and the resistant phenotype was also retained in protoplasts prepared from resistant cells (Mendoza et al. 1999).

The circular bacteriocins are probably the most stable bacteriocins known (Cobos et al. 2001). Thus far, the structure of two circular bacteriocins has been in reported. In 2000 the structure of AS-48 was determined by NMR (Gonzalez et al. 2000; Sanchez-Barrena et al. 2003), and in 2009 the three-dimensional structure of carnocyclin A (CclA) was determined (Martin-Visscher et al. 2009). These studies confirmed the unique structure of these bacteriocins, not only the C- and N-terminals covalently amide linkage, but also the remarkable similarity in their three-dimensional structures, which are made up of a compact hydrophobic core of four to five helices. It was proposed by Martin-Visscher et al. that the circular bacteriocins, despite significant differences in their primary sequences, share a common overall conserved three-dimensional structural motif of a saposin fold (Martin-Visscher et al. 2009). The saposin fold is defined among saposin-like polypeptides that include NK-lysin, a 78 amino acid residues peptide that is both antibacterial and showed a marked lytic activity against an NK-sensitive mouse tumor cell line, YAC-1, but it did not lyse red blood cells (Andersson et al. 1995; Liepinsh et al. 1997). It was also suggested that the saposin fold is likely to be a common feature of all circular bacteriocins identified to date. The structures of CclA and AS-48 enclose clusters of basic residues on the surface of the peptide, probably responsible for attracting the bacteriocins to the surface of the anionic target membrane.
The mode of action of the circular bacteriocin, carnocyclin (CclA), was determined by the use of a lipid bilayer model system (Gong et al. 2009), and it was shown that carnocyclin A forms voltage-dependent anion-selective channels in lipid bilayers (Gong et al. 2009). The channel formation was driven by the presence of a negative membrane potential that eventually led to dissipation of the membrane potential. In this perspective, it is interesting to note that the two-peptide bacteriocins plantaricin and JK also showed high conductivity for specific anions and low conductivity for cations and dissipation of the membrane potential ($\Delta\Psi$) and the pH gradient on sensitive *Lb. plantarum* cells (Moll et al. 1999b).

From the present understanding, the circular bacteriocins target the membranes of susceptible cells; however, how this takes place is not understood. It is most likely that some kind of receptor is recognized by the circular bacteriocins, as seen with other Class II bacteriocins, and through this specific interaction the circular bacteriocins somehow make the membrane permeable to low molecular weight ions, but no receptors have been identified thus far.

### 15.3.2.6 Truncated Antimicrobial Peptides

A new group of ribosomally synthesized antimicrobial peptides that we considered as being different from the regular bacteriocins are the peptides derived from larger proteins. They were first found in the eukaryotic system and were exemplified by lactoferricin derived from lactoferrin (Wakabayashi et al. 2003; Gifford et al. 2005; Jenssen 2005; Jenssen and Hancock 2009) and the antimicrobial peptide derived from histones (Park et al. 1996; Richards et al. 2001; Birkemo et al. 2003; Luders et al. 2005). Such protein-derived antimicrobial peptides have also been found in gram-positive bacteria, such as propionic acid bacteria (Faye et al. 2002; Brede et al. 2004; Faye et al. 2004), and in *Lb. reuteri* (Bohle et al. 2010). From fecal samples obtained from piglets, *Lb. reuteri* isolates were obtained and shown to produce an antimicrobial peptide named AP48-MapA. It was unequivocally shown that the antimicrobial peptide (48 amino acid residues) was a well-defined degradation product obtained from a mucus adhesion–promoting protein (Bohle et al. 2010). The AP48-MapA was able to prevent the growth of a selected number of gram-positive pathogens. This finding gives us some new perspectives of how some gut bacteria may successfully compete in this environment and thereby contribute to a healthy microbiota.

### 15.3.3 Targets and Mode of Action

The mode of action of bacteriocins has been an important topic to study, and it has been shown that the membranes of most LAB bacteriocins are targeted by these compounds. When susceptible bacteria are exposed to bacteriocins, they cause permeabilization of the susceptible cells. It was believed, and it still is, that such bacteriocins form pores or play a crucial part in membrane permeabilization and thereby give way for the free migration of ions and other small molecules across the bacterial membrane.

Lactococcin A was one of the first Class II bacteriocins to be purified and genetically characterized (Holo et al. 1991; van Belkum et al. 1991a). In an early study it was shown that it increased the permeability of the cytoplasmic membrane of susceptible *Lc. lactis* isolates (van Belkum et al. 1991). It was also shown that considerably higher concentrations of lactococcin A were needed to dissipate the membrane potential in an immune strain of *Lc. lactis*. Numerous biological transport processes were heavily affected when lactococcin A was added to bacterial cells. It was concluded that lactococcin A acts on the cytoplasmic membrane and that the interaction is very specific. The combined results obtained with cells, vesicles, and liposomes suggest that the specificity of lactococcin A might be mediated by a receptor protein associated with the cytoplasmic membrane (van
Belkum et al. 1991b). It took 16 years to identify the hypothesized receptor. In 2007, a receptor protein complex was identified as the membrane components of a mannose phosphotransferase (man-PTS) transporter (Diep et al. 2007). The mechanism, although not completely understood, probably involves an irreversible opening of the man-PTS transporter that causes a free migration of small molecules between the cytoplasm of the bacterium and its external environment.

The mechanisms behind the Class IIa bacteriocins have also been studied in detail. The initial studies performed on pediocin PA1 included how this bacteriocin affected either whole protoplasts or membrane vesicles/membrane systems (Bhunia et al. 1991; Chikindas et al. 1993; Bennik et al. 1997; Chen et al. 1997a; Chen et al. 1997b). It was generally concluded that the target for pediocin PA1 was the membrane, and that through permeabilization of the membrane the target bacterium was killed.

One study of particular interest was performed on liposomes fused with membrane vesicles derived from both sensitive and immune cells. It was shown that pediocin PA1 elicited an efflux of small ions in the sensitive system but when the vesicles of immune cells were fused, the system became much less affected by the bacteriocin. The work concluded that pediocin PA1 acts in a voltage-independent manner but that it requires a specific protein in the target membrane (Chikindas et al. 1993). Several genetic studies followed, which strongly suggested that the man-PTS system was involved in the activity of Class II bacteriocins (Gravesen et al. 2000; Dalet et al. 2001; Hechard et al. 2001; Gravesen et al. 2002b; Arous et al. 2004; Lun and Willson 2005). It was finally shown that Class IIa bacteriocins form a strong complex with its cognate immunity protein and the membrane components of man-PTS of sensitive cells, immobilizing the bacteriocin and preventing it from permeabilization and killing (Diep et al. 2007). Like lactococcin A, Class IIa also kills bacteria through interactions with the permease part of man-PTS, but the recognition of the bacteriocins and their dedicated man-PTS is very specific (Kjos et al. 2010b). While lactococcin A needs a specific part of both membrane components, Class IIa only needs one sequence-specific region of only one of the cognate components (Kjos et al. 2009; Kjos et al. 2010a).

Surprisingly, such different bacteriocins such as the strong antilisterial Class IIa bacteriocins and the lactococcal-specific lactococcin A both use the membrane part of man-PTS permease, but they are quite selective in their selection. In *L. monocytogenes* four man-PTS systems have been identified but only one of them serves as target for these bacteriocins (Kjos et al. 2009). Lactococcin A has specificity for the only man-PTS entity found in *Lc. lactis* and consequently it only kills *Lc. lactis* strains. It is of great biological interest to observe that two different groups of bacteriocins (pediocin-like bacteriocins and lactococcin A) with completely different target specificities have actually developed different parts of the same molecules for recognition.

The two-peptide bacteriocins (Class IIb) have also been shown to render the membrane of susceptible bacteria permeable to small molecules (Abee et al. 1994; Moll et al. 1996; Marciset et al. 1997; Moll et al. 1998; Moll et al. 1999a; Moll et al. 1999b; Moll et al. 2000; Castellano et al. 2003; Nissen-Meyer et al. 2010). Target specificity has yet not been identified but based on previous experience, it seems likely that their activity requires a defined target. It is also of interest to note that the two-peptide bacteriocins possess an optimal activity at a peptide ratio of 1:1 (Nissen-Meyer et al. 1992; Moll et al. 1999b), and rigorous testing of the individual peptides has found that they are not active or that they have very low antimicrobial activity. From our present knowledge, it seems unlikely that these bacteriocins act by dissolving the membrane, as suggested by many eukaryotic antimicrobial peptides, but rather that they act by using a specific target to render the membrane permeable.

We believe that the heterogeneity in the choice of targets and the difference in potency of many bacteriocins is probably due to variation in the structure of receptors (amino acid heterogeneity...
Antimicrobial Components of Lactic Acid Bacteria

and/or poor access to the receptors even within the same species of targeted bacteria). Accessibility to the receptor could be altered by changes in surface charges, membrane composition, hydrophobicity, capsular structure, and polysaccharide production.

Among the LAB lantibiotics, nisin has been the model system that has attracted the most attention when it comes to mode-of-action studies. It was shown early on that type A lantibiotics exert their antibacterial action by the formation of transient pores in the energized cytoplasmic membrane of a sensitive bacterium, leading to dissipation of the membrane potential and a rapid efflux of small metabolites and resulting in cell death, and this hypothesis was supported by a number of artificial membrane system studies (Sahl et al. 1987; Driessen et al. 1995; Sahl and Brandis 1982; Ruhr and Sahl 1985; Abee et al. 1995; Demel et al. 1996; Breukink et al. 1998; Moll et al. 1999b). It was also shown that nisin affects cell wall biosynthesis through interactions with lipid II, but this observation was not given that much attention before Sahl and his co-workers (1987) provided data on a specific interaction between nisin and lipid II (Reisinger et al. 1980; Brotz et al. 1998).

These results could explain some of the apparent discrepancies and connect the two models of nisin action in being a pore former as well as cell wall biosynthesis inhibitor. Nisin has been shown to bind to the bactoprenyl pyrophosphate moiety in lipid II, a crucial entity in peptidoglycan synthesis and assembly. Nisin can both permeabilize the membrane and inhibit the cell wall biosynthesis machinery (Bierbaum and Sahl 2009). Lipid II is then referred to as the docking molecule of nisin. The presence of lipid II in liposomes dramatically lowers the concentration of nisin needed to make liposome leaky, and the interaction with lipid II explained why nanomolar concentrations of nisin are sufficient to permeabilize cell membranes, whereas millimolar concentrations were needed to permeabilize artificial membranes (Breukink et al. 1999; Breukink and de Kruijff 2006; Hasper et al. 2006). It is now believed that nisin creates a stable membrane pore composed of eight nisin molecules and four lipid II molecules (Hasper et al. 2004). When nisin inhibits cell wall biosynthesis, lipid II becomes dislocated from the septa and the synthesis is blocked.

Many of the type A lantibiotics use lipid II as their docking molecule and they exert their antimicrobial activity through this binding site. Mutacin 1140 is another lipid II that targets lantibiotics, but it only seems able to inhibit cell wall biosynthesis as it is not able to make pores in the membrane of susceptible bacteria because it is too short (Hasper et al. 2006).

An increasing number of lantibiotics (Wiedemann et al. 2006) have been associated with lipid II, but even more surprising is that defensins (Sass et al. 2010; Schmitt et al. 2010; Schneider et al. 2010) and Class II bacteriocins (Martinez et al. 2008) have also been linked to lipid II and wall cell biosynthesis as their mode of killing.

Due to the physicochemical properties of the hydrophobic/amphiphilic properties of most antimicrobial peptides, they are able to permeabilize/dissolve membranes via a “detergent” effect and these results do not reflect their biological killing mechanisms. Both lantibiotics and Class IIa bacteriocins work well in artificial membrane/liposome systems but usually at higher concentrations than observed with targeted bacteria. Accordingly, the results of mode-of-action studies performed on other membranes and liposome systems should be interpreted with care. Presently, it seems highly likely that most bacteriocins recognize specific receptors in their initial action.

15.3.4 Regulation of Bacteriocin Biosynthesis

Cell density–dependent gene expression appears to be widely spread in bacteria and a variety of processes are known to be regulated in a cell density– or growth phase–dependent manner in gram-positive bacteria. Such a type of regulation has also been found to control the biosynthesis of
some bacteriocins by a two-component regulatory system (often referred to as a three-component regulatory system) (Diep et al. 1995; Kuipers et al. 1995) and numerous reviews on this topic have been published (Nes et al. 1996; Nes and Eijsink 1999). The three entities directly involved in the signaling pathways leading to bacteriocin production comprise (i) the signal peptide (peptide pheromone, PP), and (ii) the membrane-embedded receptor/target for the PP that upon its bidding leads to a phosphorylation signaling cascade that eventually directs a phosphate residue to (iii) the response regulator that then binds to its cognate promoters, thereby inducing gene expression and bacteriocin production. Among certain regulated lantibiotics such as nisin and subtilin, it has been shown that these bacteriocins also operate as the PP and induce their own synthesis (Kuipers et al. 1995; Kleerebezem et al. 1999), while the PPs of the regulated nonlantibiotic bacteriocins (Class II) are small specific peptides that exclusively serve a role in induction (Diep et al. 1995; Worobo et al. 1995; Diep et al. 1996; Eijsink et al. 1996; Nilsen et al. 1998). In this case the three genes are usually found in an operon structure, but other constellations exist. The regulatory system of the plantaricin system has been the most thoroughly determined, but it is also quite unique since two response regulators are found here (Diep et al. 1995; Diep et al. 1996). Experimental results surprisingly suggest that the two highly homologous response regulators (PlnC and PlnD, which are >75% similar) display totally opposite functions. While the overexpression of plnC activated transcription and bacteriocin production, the overexpression of plnD repressed both of these processes. Such a negative regulation has not been observed in other regulatory systems of bacteriocin production, but it should also be emphasized that the plantaricin system is the only known system thus far that holds two response regulators (Diep et al. 2001). The quorum sensing system includes the regulation of several operons such as itself, one or several bacteriocin, transport operon, and others. In the plantaricin system five operons have been identified, including at least two bacteriocin operons, and altogether at least 20 genes have been found to be under this regulation (Diep et al. 2009b). A detailed transcription study was performed on the regulation of the five plantaricin operons (Diep et al. 2003). The plantaricin system has been identified in a number of lactobacilli, but with some variation. Many of the bacteriocin genes are found to be common in the same or different bacterial species; however, often some bacteriocins are lost while new ones are entering the system. Figure 15.3 summarizes the various constellations of the plantaricin gene cluster that have been found thus far in lactobacilli. It is commonly accepted that many bacteria cluster multiple bacteriocin genes that share a transport system and also a three-component regulatory system as seen with the plantaricin. One may speculate if the development of new bacteriocins takes place when multiple bacteriocin genes meet in a new host and clusters in order to make the host more competitive in their fight to survive in the complex biological environment.

The five (pln) operons involved were differentially expressed with respect to both timing and strength, and the peptide pheromone triggered a strong autoactivating loop of the regulatory unit (plnABCD) during an early stage of induction that gradually led to a strong activation of the other operons. The transport operon (plnGHSTUV), which is involved in secretion of the pheromone and bacteriocins, was also expressed relatively early upon induction but it was quickly turned off soon after peak expression, probably because it was only needed in catalytic amounts. Further investigations of the various promoters revealed that although subtle differences within the promoter regions could account for the observed differential regulation, the presence of a downstream promoter-proximal sequence in one promoter caused delayed peak activity. How phosphorylation regulates the activity of the pln response regulators was also assessed by direct mutagenesis at their phosphorylation sites. It was found that the two response regulators were active on two different levels: a low level when they were not phosphorylated and an elevated level when they were
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In another study the binding site of the response regulators of the plantaricin system were scrutinized. The two inverted repeats that had been identified in the –80 to –40 region (Nes et al. 1996) were systematically mutagenized, and binding studies were performed. The binding site for the two Pln regulators consists of two 9-bp direct repeats separated by a 13-nucleotide sequence that is found in the promoter-proximal sequence in the \(\text{pln}\) regulon. This conserved promoter arrangement is found among many three-component bacteriocin regulatory systems. Point substitutions in the consensus sequence, particularly those in invariant positions, either abolished or significantly weakened the binding of PlnC and PlnD to their cognate binding sequences (Risoen et al. 1998). Both DNase I footprinting and reporter expression systems consisting of one of the regulatory promoters in conjunction with the \(\beta\)-glucuronidase (GUS) gene confirmed the dependence on an intact set of accurately organized repeats for response regulator binding and gene expression (Risoen et al. 2001). A study of another similar regulatory system confirmed how promoters involved in the quorum sensing-based regulation of sakacin P production in \(Lb. \ sake\) also differ in strength, leakiness, and timing of their activities. The sakacin P bacteriocin operons were strictly regulated, and their activity became increased by more than 1000-fold upon activation (Risoen et al. 2000).

Figure 15.3 Mosaic structure of plantaricin (pln) loci from \(Lb. \ plantarum\) strains C11 (Diep, Havarstein et al. 1996; Kleerebezem, Boekhorst et al. 2003), WCFS1 (Kleerebezem, Boekhorst et al. 2003), V90 (Diep, Straume et al. 2009), ATCC 14917 (Baylor College of Medicine), ST-III (Wang, Chen et al. 2011), BFE5042 (Cho, Huch et al. 2010), J51 (Navarro, Rojo-Bezares et al. 2008), NC8 (Maldonado, Jimenez-Diaz et al. 2004), J23 (Rojo-Bezares, Saenz et al. 2008), JDM (Zhang, Liu et al. 2009), and PCS20 (Cho, Huch et al. 2010). Loci of C11, WCFS1, V90, ST-III, and BFE5042 are identical, except (i) in V90 a transposon (indicated by a triangle) is found upstream of \(\text{plnA}\) and (ii) in V90 and BFE5042, \(\text{plnS}\) and \(\text{plnT}\) appear as a single gene. PCS20 locus is interrupted by a transposon (indicated by a triangle) in the middle of \(\text{NC8-HK}\) gene and this locus also contains an unusual \(\text{plnE}\) gene. Regulated promoters are indicated by lollipops.

phosphorylated (Diep et al. 2003). In another study the binding site of the response regulators of the plantaricin system were scrutinized. The two inverted repeats that had been identified in the –80 to –40 region (Nes et al. 1996) were systematically mutagenized, and binding studies were performed. The binding site for the two Pln regulators consists of two 9-bp direct repeats separated by a 13-nucleotide sequence that is found in the promoter-proximal sequence in the \(\text{pln}\) regulon. This conserved promoter arrangement is found among many three-component bacteriocin regulatory systems. Point substitutions in the consensus sequence, particularly those in invariant positions, either abolished or significantly weakened the binding of PlnC and PlnD to their cognate binding sequences (Risoen et al. 1998). Both DNase I footprinting and reporter expression systems consisting of one of the regulatory promoters in conjunction with the \(\beta\)-glucuronidase (GUS) gene confirmed the dependence on an intact set of accurately organized repeats for response regulator binding and gene expression (Risoen et al. 2001). A study of another similar regulatory system confirmed how promoters involved in the quorum sensing-based regulation of sakacin P production in \(Lb. \ sake\) also differ in strength, leakiness, and timing of their activities. The sakacin P bacteriocin operons were strictly regulated, and their activity became increased by more than 1000-fold upon activation (Risoen et al. 2000).
A completely different regulation mechanism is seen in the production of lactocin S, a lantibiotic produced by *Lb. sakei*, which is often found in fermented meat products (Mortvedt and Nes 1990; Mortvedt et al. 1991). The gene cluster encoding lactocin S is organized into two oppositely orientated operons, *lasAMNTUVJW* (*lasA-W*) and *lasXY*. While the nine-gene operon contains the biosynthetic machinery including lactocin S, immunity, modification, and transport genes, the two-gene operon appears to be involved in the transcriptional regulation of both operons. It has been shown that inactivation of *lasX* abolished lactocin S production by inhibiting the transcription of the lactocin S-encoding (*lasA-W*) operon. Studies of the divergently orientated and overlapping promoters of the two operons by use of the reporter gene in the presence and absence of *lasX* observed that LasX stimulated the *lasA-X* promoter and directed the expression of the reporter by 5- to 6-fold, whereas expression of the reporter, directed by the divergent promoter, was reduced by 1.5- to 2-fold (Rawlinson et al. 2002; Skaugen et al. 2002; Rawlinson et al. 2005). In other words, LasX is a bifunctional regulatory protein that acts as both an activator of *lasA-W* transcription and as a repressor of *lasXY* transcription. LasX belongs to the Rgg family of pleiotropic transcriptional activators. This family is well represented among enterococci, lactococci, and streptococci, where they regulate various functions, including the production of mutacin II, a lantibiotic produced by *Srr. mutans* (Qi et al. 1999).

### 15.3.5 Bacteriocins Resistance and Adaptation

Although bacteriocins are among the most potent antibacterial compounds known, we can see a great variety of susceptibility within them (Bennik et al. 1997), and also within species, as demonstrated for four bacteriocins and their variations in potency when three Class IIa bacteriocins and nisin were tested (Katla et al. 2003). This study showed that the susceptibility to nisin varied by approximately 390 ng/ml (between 2 and 781 ng/ml) between the 200 natural isolates of *Li. monocytogenes* tested. For the three Class IIa bacteriocins, the MIC values varied close to 100-fold between the most sensitive and the least sensitive *Li. monocytogenes* isolates. Sakacin P ranged from 0.01 to 0.61 ng/ml, and pediocin PA1a and sakacin A varied between 0.10 and 7.34 and 0.16 and 44.2 ng/ml, respectively. Nature has developed different susceptibilities to bacteria within the same species when it comes to bacteriocins; however, we do not know the reason for this intrinsic variation in resistance.

However, there have been several studies that tried to pinpoint the biological background for different bacteriocin susceptibilities. It was observed that bacteria challenged by most bacteriocins actually develop resistance but at different frequencies and to different levels. Class IIa bacteriocins are the best documented ones for creating resistant bacteria at a high frequency (Rekhif et al. 1994; Casaus et al. 1997; Dykes et al. 1998; Vadyvaloo et al. 2002; Vadyvaloo et al. 2004).

Studies have shown that specific genes in a bacterium can contribute to the intrinsic resistance in a bacterium, and presently three types of genes have been found to participate in such innate resistance: genes that encode components responsible for the cell envelope; genes that encode elements of various transporters; and regulatory genes, in particular, two-component system genes. It has been shown that alterations in the fatty acid composition in both *Clostridia* and *Listeria* make these bacteria more resistant to nisin as well as provide cross-resistance to other bacteriocins (Mazzotta et al. 1997; Crandall and Montville 1998; Vadyvaloo et al. 2002; Vadyvaloo et al. 2004). Nisin-resistant *Lc. lactis* isolates can display a significant increase in cell wall thickness of the septum. This thickening was correlated with modifications in lipoteichoic acid by increases in D-alanyl esters and galactose as substituents in the cell wall, consequently resulting in less negative charges. This was proposed as being a major defense mechanism of *Lc. lactis* against nisin (Kramer...
et al. 2008). Experimental evidence to show that the penicillin-like binding protein can contribute to nisin-resistant isolates of \textit{Li. monocytogenes} was also published (Gravesen et al. 2001; Gravesen et al. 2004).

Also, Class II bacteriocin resistance can be manifested through alterations in membrane composition (Limonet et al. 2002; Sakayori et al. 2003; Limonet et al. 2004). The ABC transporters can be part of the innate immunity of several lantibiotics and also some Class II bacteriocins (Gajic et al. 2003; Criado et al. 2006). The genes encoding the ABC transporter immune system of many lantibiotics is commonly referred to as \textit{lanEFG}. Studies of intrinsic nisin resistance were studied in \textit{Li. monocytogenes} and quite recently it was shown that a gene encoding the permease part of an ABC transporter was responsible for nisin resistance. It was shown that deletion of this gene made \textit{Li. monocytogenes} 2- and 4-fold less resistant to nisin and galidermin, respectively (Collins et al. 2010a). The mutation in this permease also made the strain more susceptible to a number of other antimicrobial compounds, such as bacitracin (8-fold). A gene probably encoding tellurite resistance (based on homology analysis) has also been shown to be involved in the innate resistance of nisin, as well as other antimicrobials. The reduction in innate nisin resistance was 4-fold in the \textit{telA} deletion of \textit{Li. monocytogenes} (Collins et al. 2010b). Again, it should be emphasized that both \textit{telA} and \textit{anrB} are proteins that generally protect the bacterium against a multitude of antimicrobials, including some lantibiotics.

Regulatory molecules have also been shown to be involved in bacteriocin resistance, and this is not surprising since they control other functional genes including the genes that affect innate immunity. It has been shown that mutations in the \textit{σ}-54 transcription factor and the activator \textit{ManR} of \textit{Li. monocytogenes} made it resistant to Class IIA bacteriocins (Gravesen et al. 2002b). It is known that these two regulatory proteins are needed to actively transcribe the receptor of these bacteriocins and consequently the bacteria became resistant when the receptor was not made (Diep et al. 2007). The general stress response factor \textit{σB} controls a number of stress genes, and when \textit{σB} is mutated in \textit{Li. monocytogenes} it becomes more susceptible to nisin (Begley et al. 2006). Two-component regulatory systems have also been demonstrated to be involved with bacteriocin resistance. Two histidine kinases (one of the proteins of the two-component systems), \textit{S} and \textit{LisK}, have been shown to influence the innate immunity of \textit{Li. monocytogenes} to nisin (Cotter et al. 2002).

Spontaneous resistance to bacteriocins is normally seen when sensitive bacteria are exposed to bacteriocins. In particular, Class IIA bacteriocin resistance to \textit{Li. monocytogenes} has shown a frequency of between $10^{-6}$ and $10^{-4}$ (Rekhif et al. 1994; Casaus et al. 1997; Gravesen et al. 2002a). It seems as though most of the resistance to Class IIA bacteriocins has to do with their receptors, the membrane-embedded part of the man-PTS system and the regulation of its expression (Gravesen et al. 2002b; Ramnath et al. 2004; Opsata et al. 2010). A detailed transcriptional analysis of how Class IIA bacteriocin resistance through a mutation of the \textit{man-PTS} affects the transcriptome of \textit{E. faecalis} has recently been published (Opsata et al. 2010). Also, resistance to nisin is commonly seen and it was reported that \textit{Li. monocytogenes} developed resistance at a frequency of between $10^{-2}$ and $10^{-7}$ upon exposure to nisin (Gravesen et al. 2002a). On the other hand, some bacteriocins seem to be consistently more potent and develop resistance much less frequently, as seen for traditional antibiotics. For example, lacticin 3147, a two-peptide lantibiotic that is produced by \textit{Lc. lactis} and efficiently kills \textit{Lc. lactis} IL1403, creates resistant colonies of IL1403 at a frequency between $10^{-9}$ and $10^{-8}$ only (Guinane et al. 2006). These isolates with low resistance could be made more resistant through exposure to increasing concentrations of bacteriocins in a stepwise manner. It has also been reported that resistance to one bacteriocin also affects the susceptibility to other bacteriocins (Naghmouchi et al. 2007).
15.3.6 Application of Bacteriocins and Bacteriocin-Producing LAB

It is important to realize that bacteriocin-producing LAB have been part of our diet for as long as LAB-fermented food has existed. Most bacteriocinogenic LAB are actually isolated from food, and it is difficult to see how we can keep them away from our daily diet. In addition, our intestinal LAB flora also includes potential bacteriocin producers, as has been shown in several studies where bacteriocin-producing LAB have been frequently found in the digestive systems and fecal samples of humans and animals.

Many reviews have covered the broad field of bacteriocin applications (Delves-Broughton et al. 1996; Ennahar et al. 1999; Cleveland et al. 2001; Ross et al. 2002; Kirkup 2006; Settanni and Corsetti 2008; Garcia et al. 2010; Pieterse and Todorov 2010), and in spite of the strong focus on applications the lack of success is noticeable. Bacteriocins can be incorporated into food in order to extend or improve the shelf life by various means. Bacteriocin-producing LAB can either be used as a starter culture in a fermented product or they can be added to fresh food as a protective culture. Alternatively, bacteriocin preparations can be used as an ingredient in food, as seen with the commercialized Nisaplin\textsuperscript{TM}, a concentrate of nisin delivered in a nonfat milk base.

Bacteriocins are used as food preservatives with limited success, but nisin is an exception because its use is accepted in certain food products in more than 50 countries and in many food commodities. Other bacteriocin products have also been launched on the food market in order to improve food safety, but not with the same level of success. However, a greater knowledge of how they work and how to deliver such compounds to food and new bacteriocins should give hope for more successful applications. Medical-related application of bacteriocins has been found useful. Nisin as well as lactacin 3147 has been shown effective in protecting against bacterial mastitis as well as in treating mastitis (Broadbent et al. 1989; Ryan et al. 1998; Twomey et al. 2000).

Probiotic food has been become very popular in recent years. Such products are mainly enriched by living bacteria, such as bifidobacteria and lactobacilli, which are claimed to improve health and increase resistance to infectious bacteria (Ouwehand et al. 2002). Such bacteria are sold as a microbial supplement with beneficial effects to the consumers. Most probiotics fall into the group of lactic acid–producing bacteria and are normally consumed in the form of yogurts, fermented milks, or other fermented foods (Ouwehand et al. 2002; Ouwehand and Vesterlund 2003; Isolauri et al. 2004; Meurman 2005; Sullivan and Nord 2005; Parvez et al. 2006; Collado et al. 2009; Fung et al. 2009), and it has been shown that many probiotics can also produce bacteriocins in commercially available cultures. The open question is, are these potential bacteriocin producers able to actually produce the bacteriocins in the intestinal environment? Recently, an elegant experiment was carried out on feeding mice with the bacteriocin-producing probiotic \textit{Lb. salivarius}, followed by the administration of \textit{Li. monocytogenes}. Whereas the control mice died, the mice that received the bacteriocin producer survived (Corr et al. 2007). When the \textit{Li. monocytogenes} pathogen was made immune to the bacteriocin, the mice were not protected by the bacteriocin-producing probiotic and died. This experiment conclusively showed that the active entity that protected the mice against the \textit{Li. monocytogenes} infection was the bacteriocin. These findings provide high hopes for the production of bacteriocin-producing probiotics and applications of bacteriocins for protecting or even combating bacterial infections.

15.3.7 Additional Aspects Concerning Bacteriocins

The revelation of the complex biosynthesis machinery of lantibiotics has made them attractive for applying peptide modification enzymes in the synthesis of novel molecules and proteins. Through
such a strategy, one should be able to make molecules with unique properties (e.g., protease resistance entity, antimicrobial compounds, and new drugs) for industrial and medical applications.

Also, the chemical synthesis of peptides is an established technique, and it is often easier to chemically synthesize unmodified bacteriocins than to use the processes of fermentation and purification. This is not the case with lantibiotics, but, recently, Ross and co-workers performed solid-support synthesis of active lactocin S, a lantibiotic produced by a *Lb. sakei* strain (Ross et al. 2010). This peptide contains two lanthionine structures as well as four dehydrated residues (two Dha and two Dhb) (Ross et al. 2010).

The two (or three)-component regulatory systems of bacteriocins have been developed for application in gene cloning to control gene expression; in particular the nisin induction system has been used extensively in regulating gene expression (de Ruyter et al. 1996; Kleerebezem et al. 1997). In addition the regulatory system of sakacin A and sakacin P has been found useful in regulating gene expression of cloned genes (Axelsson et al. 1998; Sorvig et al. 2005; Diep et al. 2009a).

### 15.4 Concluding Remarks

The antimicrobial potential of lactic acid is unique among living microorganisms, and it also represents a great potential in application that still is in its infancy. However, to fully exploit its possibilities, considerable intellectual and economical resources have to be invested in such an effort. In a time when we are seeing increasing problems with the use of traditional antibiotics, other compounds have to be investigated more thoroughly and we have to look on such means differently from how we see it today. Compounds that specifically target the pathogen and do not affect other and useful organisms may be advantageous for the host (human or animal), as has been reported for thuricin CD that is targeting *Cl. difficile* and leaves the human microbiota mostly unaffected (Rea et al. 2010). Probiotic bacteria will also benefit from being a bacteriocin producer; this was convincingly demonstrated in mice where they were protected by such a bacterium against *Li. monocytogenes* infection (Corr et al. 2007).

The effects of bacteriocins and bacteriocin-producing LAB have been extensively investigated in many food commodities and food simulated test systems, and with good effects but apparently not sufficient to convince the food producers to include them into foods. Maybe the expectation is too high. When it is observed that the extension of shelf life of the food or the reduction of spoilage organisms or potential pathogens in the food is not sufficiently great, it will cost too much to use such technologies. The future will certainly bring new and improved systems for delivery of bacteriocins into food, which will make their application in food more favorable.

Still we are only seeing the tip of the iceberg when it comes to the diversity and multiplicity of bacteriocins in nature, and scrutinizing new bacterial genome sequences will be the most efficient and productive way to find new entities. In addition, protein engineering will be an important means to create new antimicrobial molecules that might be used to fight pathogens and spoilage microorganisms.

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Antimicrobial Components of Lactic Acid Bacteria


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Chapter 16

Atherosclerosis and Gut Microbiota: A Potential Target for Probiotics

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16.1 Introduction

Cardiovascular diseases (CVD) are the number one cause of death globally: more people die annually from the disease than from any others (Loscalzo 2005). About 17.1 million people were estimated to have died from the disease in 2004, and it is estimated to increase to 23.6 million by 2030. The majority of CVD deaths are due to heart disease and stroke, for which the main cause is atherosclerosis (World Health Organization [WHO] 2011).

Atherosclerosis is a chronic inflammatory disease resulting from compromised ability to resolve inflammatory response to subendothelial lipoproteins (Mach 2005). An atherogenic environment results in endothelial dysfunction, which allows the crossing of bad cholesterol—low density lipoprotein (LDL)—and be oxidized to oxLDL, which leads to a sequence of inflammatory response and result in the buildup of atherosclerotic plaque that occlude a blood vessel (Frederick and Ruberg 2005).

The human gut is inhabited by up to 100 trillion microbial organisms, collectively known as the microbiota. The microbiota participate in the regulation of cholesterol metabolism, while several research studies have revealed a close relationship of gut microbiota with inflammation and obesity and obesity-related comorbidities such as type II diabetes (DiBaise et al. 2008; Musso et al. 2011). Since lipidemia, diabetes, and obesity are crucial and well-known risk factors in the inflammatory process of atherosclerosis, and the alteration of the gut microbiota has been shown to affect the disease progression of these risk factors, in this chapter we seek the potential significance of the relationship of the gut microbiota and atherosclerosis.

16.2 Atherosclerosis—Pathogenesis

Atherosclerosis is commonly referred to as a “hardening” or “furring” of the arteries with formation of multiple plaques that narrow the arterial passage, restricting blood flow, and predisposes to hypertension and deficient blood and oxygen supply (ischemia) in the tissues served by the artery. Ischemia is the primary cause of atherosclerosis-associated complications such as coronary heart disease and stroke. The development of atherosclerosis is a slow process, which can occur over the course of 50 years beginning at the teenage years (Insull 2009). While CVDs can be considered a problem of high-income countries following the Western lifestyle, in fact more than 80% of the world’s deaths from CVDs occur in low- and middle-income countries. This is because they are more exposed to the risk factors for CVDs and other noncommunicable diseases, less exposed to prevention efforts, and have less access to effective and equitable health-care services. Furthermore, these countries are acquiring the proatherogenic habits of the Western world, WHO predicts that atherosclerotic vascular diseases are becoming a truly global epidemic. From an economical point of view, CVD place a heavy burden in all countries, especially for low- and middle-income countries.

There are several known risk factors for atherosclerosis of which many are definitely modifiable, such as blood total cholesterol (TC), high-density lipoprotein (HDL) and triglyceride (TG) concentrations, blood pressure, diabetes, obesity, sedentary lifestyle, smoking, and alcohol intake (Wilson 2000). The major mechanisms that contribute to the development of atherosclerosis involve endothelial dysfunction, inflammation, hyperlipidemia, oxidative stress, thrombosis, and excess proliferation of vascular smooth muscle cells. Inflammation participates in all stages of atherosclerosis, not only during initiation and during evolution of lesions but also with precipitation of acute thrombotic complications. Understanding the development of atherosclerotic plaque is very crucial in the attempt to its prevention.
16.2.1 Formation of Atherosclerotic Plaques

Blood vessel is composed of three layers. The outer layer is mostly connective tissues; the middle layer is mainly smooth muscles, while the inner lining consists of a thin layer of endothelial cells (EC) or the endothelium. ECs are the central regulators of inflammation interacting with the different leukocytes such as monocytes, polymorphonuclear neutrophils, and B and T lymphocytes, expressing a vast array of surface receptors and releasing signaling molecules, including inflammatory cytokines and adhesion molecules (Ruberg 2005).

When the ECs are exposed to atherogenic environment that may result, for example, from dyslipidemia, diabetes, smoking, hypertension, physical inactivity, bacterial infection, or increased level of angiotensin II, the ECs will undergo a transformation referred to as endothelial dysfunction. There will be an increase in endothelial permeability to LDL. LDL will thus cross the endothelial barrier and is oxidized (oxLDL) by the reactive oxygen species (ROS) produced by the EC and other leukocytes, including neutrophils and macrophages. oxLDL stimulates dysfunctional EC to release pro-inflammatory chemokines such as monocyte chemoattractant protein-1 (MCP-1) and growth factors such as monocyte colony-stimulating factor (mCSF), as well as expression of cell adhesion molecules including vascular-cell adhesion molecule 1 (VCAM-1), intercellular cell adhesion molecule 1 (ICAM-1), and E-selectin resulting in recruitment of leukocytes, monocytes, and T lymphocytes in particular. The gene expression of pro-inflammatory molecules such as tumor necrosis factor $\alpha$ (TNF$\alpha$), interleukin 1$\beta$ (IL-1$\beta$), IL-6, MCP-1, and ICAM-1, are all controlled by the nuclear factor kappa B (NF$\kappa$B) pathway (Baker et al. 2011).

During endothelial dysfunction, the ability of EC to produce nitric oxide (NO), a central physiological homeostatic regulator of the vessel walls, is also impaired. The antiatherogenic ability of NO to inhibit monocyte adhesion to the endothelium (Tsao et al. 1996), inhibit expression of VCAM-1 (Lee et al. 2002) and MCP-1 (Desai et al. 2003), and to reduce the activity of NF$\kappa$B (Lee et al. 2002) is thus reduced.

The expression of adhesion molecule can be further up-regulated by pro-inflammatory cytokines produced by the activated ECs and macrophages, such as IL-1 and TNF$\alpha$, as well as being induced by lipopolysaccharides (LPS) (Liao 1996). The infiltrating leukocytes enhance the recruitment of their counterparts by producing more chemokines such as MCP-1, macrophage inflammatory protein 1 (MIP-1), and IL-8, and activating their production by smooth muscle cells (Mach 2005).

Monocytes penetrate the endothelium and turn into macrophages. The macrophages express scavenger receptors that aggressively uptake oxLDL to become cholesteryl ester–rich foam cells (Matsumoto et al. 1990). NF$\kappa$B takes an active role in plaque formation and is essential in controlling the survival of foam cells (Kanters et al. 2003). Foam cells release growth factors that further stimulate proliferation of vascular smooth muscle cells (VSMCs). During the progression of the plaque, some foam cells die and become nidus of the necrotic core (Pang et al. 1999). The accumulation of foam cells and proliferation of VSMCs result in plaque growth.

16.2.2 Thrombosis

With the continuous progression of atherosclerotic plaque, the plaque is at risk of disruption. When there is fissure or rupture in the plaque, thrombogenic materials in the lesion interact with blood coagulation factors (Mach 2005), leading to thrombus formation. When a plaque has outward vessel remodeling, a large lipid core, thin fibrous cap, reduced collagen content, and increased number of inflammatory cells, it is at risk of rupture. Activated macrophages produce
increased amount of proteases, including metalloproteinase 2 (MMP-2) (Li et al. 1996), MMP-9 (Brown et al. 1995; Gottschall et al. 1995), and other connective tissue enzymes that can break down collagen and weaken the fibrous cap. Depending on the site of thrombotic occlusion, or the site where the plaque has narrowed the blood vessel to a state where the organ blood supply is no longer sufficient, ischemic heart disease, kidney failure, or stroke would take place.

### 16.3 Gut Microbiota and Atherosclerosis

Chronic low-grade inflammation is the key that links metabolic disorders, including atherosclerosis, obesity, type II diabetes, and insulin resistance. NFκB is a transcription factor that controls the expression of a range of genes involved in inflammation and also a central factor in the inflammatory metabolic disorder. In obesity, inflammation leads to production of inflammatory chemokines and cytokines such as MCP-1, MIPs, IL-6, IL-1β, and TNFα from adipose disuse (Xu et al. 2003); in diabetes, inflammation of macrophages produce IL-1, IL-6, and TNFα, which promotes pancreatic β-cell dysfunction and death, disrupting the insulin signaling and triggering insulin insensitivity.

The gut microbiota contributes to gut barrier functions and can thus indirectly affect the translocation of antigenic material and define the regulatory signals welling from the gut (Izcue et al. 2009; Leser and Molbak 2009). Toll-like receptors (TLRs) compose a family of recognition receptors for pathogen-associated molecular patterns that play important roles in innate immunity (Beutler 2004). Activation of TLRs, regardless of the stimulated receptor, leads to activation of NFκB and transcriptional activation of genes that encode pro-inflammatory cytokines, chemokines, and co-stimulatory molecules. TLR activation may interfere with glucose metabolism (Raetzsch et al. 2009), insulin response (Tsukumo et al. 2007), and adiposity (Davis et al. 2008). Vascular cells are responsive to ligands of TLR5, at least transiently during the development of carotid atheroma (Erridge et al. 2008). TLR2 and TLR4 are important in the inflammation-mediated atherosclerosis. Their expression was increased in developing atherosclerotic plaques and is associated with a monocyte surface receptor (Bielinski et al. 2011). TLR3 was unexpectedly found to have a protective effect on the vessel wall (Cole et al. 2011). Although the exact interaction of TLR in the development of atherosclerosis is yet to be defined, it is certain that it has a vital role in the process.

Recently, several reports highlighted that in addition to the increase in plasma concentration of the inflammatory molecules, a moderate increase in the bacterial LPS was observed during a fat-enriched diet, and defined metabolic endotoxemia (Cani et al. 2007b; Cani et al. 2008). LPS is responsible for the onset of metabolic diseases (Cani et al. 2008) because a continuous subcutaneous low-rate infusion of LPS can induce most, if not all, of the features of metabolic diseases, implying its potential role as a causative agent. It was noted that there were changes in the intestinal microbiota, with the ratio of gram-negative to gram-positive bacteria being increased during a high-fat diet (Cani et al. 2007b). Dietary fibers have been shown to normalize the gram negative-to-gram positive ratio and plasma endotoxemia (Cani et al. 2007a) and reduced the impact of high-fat diet on the occurrence of metabolic diseases (Cani et al. 2006). These data implied that intestinal microbiota could be responsible for the changes that lead to metabolic endotoxemia and for the onset of the corresponding diseases, although the causative link between intestinal bacteria, endotoxemia, and metabolic disease has not been demonstrated.

One of the potential molecular pathways associating the microbiota and the host is the AMP-activated protein kinase (AMPK) pathway. AMPK is a key enzyme that controls cellular energy status. Germ-free mice fed with a high-fat, high-sugar Western diet remained lean despite a high calorie intake. This was associated with increase in AMPK phosphorylation in the liver and skeletal
muscle, where fatty acid oxidation in peripheral tissues was simulated and lead to decreased glyco-
gen levels in liver. AMPK has been reported to suppress oxLDL-induced macrophage proliferation
and inhibit oxLDL-triggered ER stress in vivo (Ishii et al. 2009). AMPK can alleviate atherosclerosis in ApoE−/− mice by inducing cholesterol efflux from foam cells (Li et al. 2010). When AMPKα2
is reduced, ER stress and atherosclerosis were shown to be increased in vivo (Dong et al. 2010). If
gut microbiota has the potential to increase AMPK phosphorylation activity, then it poses the pos-
sibility of having an antiatherogenic property. Although until recently there are only a few related
studies, the following cases do support the idea.

When ApoE−/− mice, an animal model that develop hyperlipidemia and atherosclerotic plaque
readily with all phrases of atherosclerotic lesions that are seen in humans (Zhang et al. 1992), were
fed with prebiotics long-chain inulin, the atherosclerotic lesion size decreased by 35% (Rault-
Nania et al. 2006). Where germ free and conventionalized ApoE−/− mice were fed a low-cholesterol
diet, the size reduction in the atherosclerotic plaque was significant; however, when a hypercholes-
terol diet was used instead, the size reduction was not obvious. On the other hand, under either
diets, the conventionalized mice consistently showed a significant lowered blood cholesterol level.
This experiment showed that the vital influence of commensal microbiota formation of athero-
sclerotic plaque and blood cholesterol levels (Stepankova et al. 2010). Also, an experimental work
that is still in preparation by Fåk’s group demonstrated that the germ-free ApoE−/− mice were
protected from atherosclerosis when compared with the conventionalized ones under high-fat diet
for 12 weeks (Caesar et al. 2010). In a recent study by Vijay-Kumar and co-workers, it was shown
that when the gut microbiota is transferred from a TLR5−/− mice, which readily develop signs of
metabolic syndrome, to healthy mice, the metabolic phenotype was also transferred (Vijay-Kumar
et al. 2010). This suggests that altering the gut microbiota composition can have major impact on
the metabolic phenotype and thereby could also affect atherosclerosis development in the host.
While the mechanisms of the above studies are yet to be determined, they strongly suggest that the
gut microbiota profile is a vital factor influencing the development of atherosclerosis.

Because of the possible potential associations of the gut microbiota and atherosclerosis as dis-
cussed above, it is quite plausible that altering the microbiota composition using pro/prebiotics
might pose a protective role in the host against atherosclerosis.

16.4 Probiotics as Potential Modulators of Atherosclerosis

As described above, several mechanisms contribute to atherogenesis. Here, we will review the
potential mechanisms by which probiotics might interfere with this process. These are primarily
centered via long-term prevention or alleviation of inflammation, for example, through fortified
gut barrier function, reducing translocation of endotoxins and other potential oro-gastrointestinal
tract originating proinflammatory agents such as bacterial components or bacteria including those
associated with periodontal disease and Helicobacter pylori infection. Probiotics may, however, also
affect blood cholesterol metabolism.

16.4.1 Effect of Probiotics in Modulation of Inflammation

Different strains of probiotics have been shown to possess anti-inflammatory properties, especially
in the treatment of some chronic inflammatory diseases such as allergies (Verma et al. 2002; Isolauri
et al. 2008) and inflammatory bowel disease (Gionchetti et al. 2000; Lorea Baroja et al. 2007).
Lactobacillus casei DN-114-001 has been shown to decrease the pro-inflammatory cytokine TNFα
secreted from the inflamed ileum of Crohn’s disease patients (Borruel et al. 2002); *Bifidobacterium breve* Yakult and *B. bifidum* Yakult has been shown to induce IL-10 production in human peripheral blood mononuclear cells of ulcerative colitis patients and inhibited pro-inflammatory cytokine IL-8 production in the HT-29 cell line (Imaoka et al. 2008); VSL#3 was shown to increase the anti-inflammatory cytokine IL-10 production by dendritic cells (Drakes et al. 2004); *L. casei* posed an anti-inflammatory effect on *Shigella*-infected human intestinal epithelial cells possibly through inhibition of NFκB activation (Tien et al. 2006); *L. plantarum* MG208, *L. rhamnosus* MG316, and *L. acidophilus* MG501 were shown to effectively inhibit inflammatory cytokines, including TNFα and IL-8, in *H. pylori* infection *in vitro* possibly by enhancing the expression of suppressor of cytokine signaling (SOCS) 2 or SOCS3 or inactivating Janus kinase (JAK) 2 through JAK-signal transducers and activation of transcription (STAT) pathways (Lee et al. 2010). In addition to the above *in vitro* studies, recently a 3-week invention of LGG in 68 human adults has been shown to lower serum highly sensitive C-reactive protein (hsCRP) (Kekkonen et al. 2008), a marker of inflammation (Volanakis 2001) and heart disease risk (AHA 2011), and also a potential candidate that may contribute to atherogenesis (Verma et al. 2002; Verma et al. 2004).

Moreover, NFκB is important in the induction of iNOS expression in both murine and human cells. The production of NO, the vasodilator, was induced by LGG by induction of inducible NO synthase (iNOS) in IFNγ primed J774 murine macrophage (Korhonen et al. 2002). The increased NO production might confer vasodilation effect in the artery.

### 16.4.2 Probiotics May Reduce Endotoxemia and Other Gut-Originating Pro-Inflammatory Antigen Burden

There are two primary mechanisms by which intestinal translocation of LPS is thought to occur (Caesar et al. 2010). LPS can be transported through enterocytes, especially via adherence to chylomicrons (Ghoshal et al. 2009), or LPS may enter the circulation via passive extracellular leakage through alterations in tight junction structure in the epithelial lining.

Some probiotics such as *L. plantarum* DSM 2648 (Anderson et al. 2010a), *L. plantarum* MB452 (Anderson et al. 2010b), LGG (Donato et al. 2010), and VSL#3 (Mennigen et al. 2009) were shown to be able to enhance the epithelial barrier function. Some studies have shown that after the improvement of tight junctions and gut barrier with probiotics, the chances of LPS leakage was decreased (Isolauri et al. 1993; Purohit et al. 2008; Mennigen et al. 2009; Anderson et al. 2010a; Anderson et al. 2010b; Donato et al. 2010; Zhou et al. 2010). LGG has been shown to reduce the plasma endotoxin levels, and with prebiotics oligofructose administration (Nanji et al. 1994), not only plasma LPS but also inflammatory cytokines were reduced (Cani et al. 2007a). Moreover, TLRs are also activated and associated with atherosclerosis (Erridge et al. 2008; Coenen et al. 2009; Bielinski et al. 2011; Cole et al. 2011); the translocation of TLR agonists such as double-stranded RNA and flagellins might then be critical to the development of atherosclerosis, especially when the gut also contain a viral microbiota (Reyes et al. 2010). If endotoxemia, or translocation of other bacterial material from the gut, the potential cause of chronic inflammation and metabolic disorder, can be reduced by improving tight function in intestinal ECs, then atherosclerosis, as a chronic inflammatory disease, might be prevented by the administration of probiotics.

### 16.4.3 Effect of Probiotics on Periodontal Disease

Periodontal diseases appear to predispose to atherosclerosis, suggesting that oral microbiota might contribute to cardiovascular disease. In one study the DNA of *Chryseomonas* was detected in all
Atherosclerotic plaque samples of the 15 patients studied, and Veillonella and Streptococcus in the majority. Several bacterial taxa in the oral cavity and the gut correlated with plasma cholesterol levels. Moreover, the amount of bacterial DNA correlated positively with the number of leukocytes within the plaque, suggesting contribution to the inflammatory status of the plaque. They suggested that bacteria from the oral cavity or even the gut may correlate with disease markers of atherosclerosis, and that the bacteria present in the plaque could be derived from the distal gut and the oral cavity, transported by macrophages that have phagocytosed them at epithelial lining (Koren et al. 2010).

Notably, probiotics have been associated with the ability to prevent periodontal diseases (Silva 1987). LGG has been shown to have antimicrobial activity against Streptococcus spp., which is known to cause tooth decay. In another study, involving nearly 600 children at municipal daycare centers, supplementation of milk with LGG nearly halved the odds of clinical development of dental caries when compared with the placebo group receiving milk only (Nase et al. 2001). Thus, probiotics might help reduce the chance of development of atherosclerosis due to periodontal disease.

16.4.4 Interaction of Probiotics and H. pylori

H. pylori infection has been shown to enhance atherosclerosis in animals fed a high-cholesterol diet. Similarly in humans, the association between CagA-positive H. pylori infection with the virulent CagA-positive strains and coronary atherosclerotic burden has been demonstrated (Niccoli et al. 2010). The H. pylori infection and atherosclerosis may be linked via the activation of CD40/CD40 ligand pathway, which is also involved in insulin resistance and type II diabetes (Eshraghian and Eshraghian 2010).

Various lactobacillus strains, including L. johnsonii La1 and L. acidophilus CRL639, have been shown to inhibit or kill H. pylori in vitro (Hamilton-Miller 2003). LGG and L. rhamnosus Lc705 have also been shown to inhibit the H. pylori adhesion and inflammatory response (inhibition of IL-8 and PGE₂ release) of the epithelial cell line Caco-2 (Myllyluoma et al. 2008). In this sense, probiotics might help reduce atherosclerosis caused by H. pylori.

16.4.5 Effects of Probiotics on Cholesterol Level

Serum cholesterol is a major risk factor of atherosclerosis, and LDL cholesterol (LDL-C), HDL cholesterol (HDL-C), and TG are definitely modifiable risk factors of coronary heart disease (Wilson 2000). 3-Hydroxy-3-methyl-glutaryl-CoA reductase inhibitors (statins) are commonly prescribed by medical doctors to patients to lower LDL and increase HDL; however, certain side effects are unavoidable (Nair et al. 2010). Thus, if probiotics have a cholesterol-lowering effect and at the same time have little known side effects, their use might be desirable as a mild preventive measure for poor lipid profile development.

Some clinical and experimental studies suggest that probiotics may have hypocholesterolemic effect, yet the issue remains controversial. The following randomized, placebo-controlled studies support the hypocholesterolemic effects of probiotics. When Sprague–Dawley rats were fed with L. plantarum CK 102 administrated at 5.0 × 10⁷ CFU/ml daily for 6 weeks, TC, LDL-C, and TG were all significantly decreased (Ha et al. 2006). When Awassi weaning lambs were fed with L. acidophilus daily at 2 × 10⁹ CFU for 120 days, TC significantly decreased (Lubbadeh et al. 1999). When L. plantarum KCTC3928 was administrated at 1 × 10⁹ CFU/ml for 4 weeks in induced hypercholesterolemic C57BL/6 male mice, TC, LDL-C, and TG significantly decreased
while HDL-C significantly increased (Jeun et al. 2009). In some clinical studies, \textit{L. bulgarius} and \textit{S. thermophilus} reduced serum cholesterol significantly (Hepner et al. 1979); \textit{L. acidophilus} L1 reduced serum cholesterol significantly (Anderson et al. 1999); \textit{L. acidophilus} 145 and \textit{B. longum} 913 increased serum HDL significantly (Kiessling et al. 2002).

Although the above studies found promising hypocholesterolemic effects of probiotics on both animals and humans, some other studies have demonstrated no effect. For example, administration of \textit{L. rhamnosus} LC705 at $2 \times 10^{10}$ CFU/day for 38 men for 4 weeks (Hatakka et al. 2008); \textit{L. fermentum} at $8 \times 10^9$ CFU/day for 46 human volunteers for 10 weeks (Simons et al. 2006); and \textit{L. acidophilus} at $9 \times 10^{10}$ CFU/day for 8 volunteers for 6 weeks (Lewis and Burmeister 2005) also did not have an effect on TC, TG, HDL-C, or LDL-C levels.

While the results above are controversial, the discrepancies might be due to the fact that there is an absence of dose–response studies to determine the “minimal effective dosage” to improve the lipid blood profile (Ooi and Liong 2010). Also while several mechanisms have been proposed for their mediating effect via \textit{in vitro} studies, they are not firmly established or demonstrated in \textit{in vivo} studies yet.

### 16.5 Preliminary Studies

In our ongoing experiments, we have studied the effects of dietary supplementation with probiotics, alone or in combination with telmisartan, on atherosclerosis formation in ApoE\textsuperscript{−/−} mice on a high-fat diet. Telmisartan is an angiotensin II type I blocker and known peroxisome proliferator-activated receptor \(\gamma\) agonist that has been proven to reduce and stabilize atherosclerosis in ApoE\textsuperscript{−/−} mice and is commonly used in the management of hypertension in humans.

Preliminary data are very promising, showing that probiotics with telmisartan appeared to be significantly more potent inhibitors of atherosclerotic lesions than the positive control telmisartan alone. Interestingly, the efficacy of the treatments appeared to be associated with differences in colonic microbiota development as demonstrated by temperature gradient gel electrophoresis–based analysis with universal bacterial primers. The microbiota composition of the most efficient treatments seemed to group close together in the principal component analysis plot; as the efficiency of the protective effect of the treatment increases, they tend to move further away from the control.

While these data strongly support the idea of using probiotics or other host–gut microbiota interaction targeting treatments in the prevention of atherosclerosis, it must be cautioned that the pilot study is at an elementary stage, and larger sample size and more detailed microbiological analyses are required to confirm the findings.

### 16.6 Conclusion

The link between gut microbiota and the metabolic status of the host is becoming increasingly evident, but still not completely understood. As the modulation of gut microbiota appears to confer beneficial effects for obesity and insulin resistance, the disorders that share many common underlying mechanisms with atherosclerosis, it is arguable that altering the gut-originating microbial stimulation in a specific manner could result in atherosclerosis-preventive or -ameliorating effects.
References


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WHO (2011) Cardiovascular Disease Fact Sheet.


Chapter 17

Lactic Acid Bacteria (LAB) in Grape Fermentations—an Example of LAB as Contaminants in Food Processing

Eveline Bartowsky

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17.1 Introduction

The production of alcoholic beverages dates back over 7000 years. Even though the concept of transforming grape juice into wine does not appear difficult, it can be a complex process to ensure an enjoyable, fault-free, and stable product. The winemaking process used today is not vastly different from that used in the time of the ancient Egyptians and Greeks. However, modern-day winemakers have much more control at the various critical stages from the time of picking grapes through to wine maturation.

Historically, early microbiologists such as Pasteur and Müller-Thurgau had observed the presence of bacteria in wine, and by early 1900, the importance of these bacteria in winemaking was beginning to be understood. They were observed in both sound and spoiled wine. The main function of lactic acid bacteria (LAB) in wine is to conduct malolactic fermentation (MLF), which is the decarboxylation of L-malic acid to L-lactic acid (Henick-Kling 1993; Möslinger 1901). This increases the wine pH by 0.2–0.5 units, resulting in a softer-tasting wine, and provides microbial stability by the removal of a potential carbon source. Moreover, through bacterial metabolism during MLF, there are various sensory changes that can occur in the wine (Lonvaud-Funel 2000).

Wine LAB are able to produce a large number of secondary metabolites, with most resulting in favorable sensory outcomes. There are several recent reviews that cover this aspect of wine LAB (Lonvaud-Funel 2000; Bartowsky 2005; Swiegers et al. 2005; Bartowsky and Pretorius 2008; Bauer and Dicks 2004; Liu 2002). This chapter will discuss the undesirable outcomes of bacterial metabolism during grape fermentations, and the various wine spoilage scenarios that can result from LAB contamination in wine.

17.2 LAB in Grape Fermentation

The presence of bacteria and the role that they play in winemaking has been known since the mid-late 1800s. Grape- and wine-associated bacteria belong to the acetic acid bacteria (AAB) and LAB families. The main role in food fermentation by the AAB is the production of acetic acid from ethanol, the basis of vinegar. Under conducive growth conditions, this family of bacteria can cause wine spoilage; they will not be discussed in this chapter, but there are several recent reviews for the reader to refer to (Bartowsky and Henschke 2008; Cleenwerck and De Vos 2008).

There are four LAB genera associated with wine: Lactobacillus, Leuconostoc, Oenococcus, and Pediococcus, and they can be readily distinguished morphologically by microscopic examination. Lactobacillus species produce short to long slender rod-shaped cells (0.5–1.2 µm × 1.0–10 µm). Leuconostoc and Oenococcus usually consist of spherical to lenticular cells, most often occurring in pairs or chains (1–2 µm diameter). Pediococcus species occur as spherical-shaped cells that are usually found in tetrads (division in two planes; 0.5–0.7 µm diameter, 0.7–1.2 µm length). The species that commonly occur in wine are Oenococcus oeni, Lactobacillus plantarum, Lb. brevis, Lb. cellobiosis, Lb. buchneri, Lb. casei, Lb. hilgardii, Lb. trichodes, Leuconostoc mesenteroides, Pediococcus pentosaceus, P. damnosus (P. cerevisiae), and P. parvulus. The taxonomic classification of LAB is discussed elsewhere in this book.
The main role of LAB in grape fermentation is conducting MLF, which generally commences as spontaneous or natural reaction about 1–3 weeks after completion of alcoholic fermentation and lasts 2–12 weeks. LAB originating from the vineyard, on the grapes, and resident in the winery are responsible for the MLF (Bae et al. 2006; Renouf et al. 2005); however, today, winemakers can choose to encourage the onset of MLF by inoculation with commercial cultures of *O. oeni*. All wine-associated LAB are able to conduct MLF; however, usually it is conducted by *O. oeni* strains. This species is well adapted to the harsh conditions of wine (low nutrients, high acidity, ethanol concentrations up to or more than 15% v/v). Other LAB genera and species are usually associated with wine spoilage, and the production of undesirable aroma and flavor compounds.

### 17.3 Spoilage of Grapes and Wine

All wine-associated LAB species will form secondary metabolites as they grow in wine. The desirability of these compounds is often concentration dependent. Types of wine spoilage are summarized in Figure 17.1 and Table 17.1. Many of the wine spoilage scenarios discussed below have been characterized over the last 50 years (Sponholz 1993; Bartowsky 2009; Bartowsky and Pretorius 2008), with few new ones being described. The types of spoilage associated with contaminating LAB in wine range from overtly buttery aromas, vinegary descriptors, through to mousy off-flavor, hints of geranium aromas or viscous wines, and compounds that may affect the consumer’s health, such as biogenic amines or ethyl carbamate.

#### 17.3.1 Volatile Phenols

Volatile phenols are formed from the hydroxycinnamic acid precursors present in grape must predominantly by yeast (*Dekkera/Brettanomyces*) during fermentation and wine maturation, and contribute to off-flavors with descriptors such as bandaid, barnyard, medicinal, or stable (Dubois 1983). Several LAB and fungi have the genes encoding phenolic acid decarboxylases (Chatonnet et al. 1995; Du Toit and Pretorius 2000). Strains of *Lb. brevis*, *Lb. collinoides*, and *Lb. plantarum* have been shown to possess the enzymes to produce 4-ethylphenol from *p*-coumaric acid (Couto et al. 2006). In the same study, pediococci strains were able to metabolize *p*-coumaric acid only through to 4-vinylphenol. Using molecular techniques, this ability of *Lactobacillus* and *Pediococcus* to convert *p*-coumaric acid to the 4-vinylphenol was confirmed by identifying the presence of the gene *pdc* (*phenol decarboxylase*) in numerous strains (De Las Rivas et al. 2009). The same study confirmed the absence of the *pdc* gene in *O. oeni* strains (De Las Rivas et al. 2009). The yeast *Dekkera/Brettanomyces* is able to efficiently conduct the final step to form 4-ethyl phenol in wine, often at very high concentrations, and thus are the main contributors to these spoilage aromas and flavors in wine (Curtin et al. 2007; Heresztyn 1986a).

#### 17.3.2 Sulfur Compounds

Sulfur-containing compounds typically occur in wine at very low concentrations, have very low sensory detection thresholds, and generally confer negative sensory attributes to wine, with descriptors such as cabbage, rotten egg, sulfurous, garlic, onion, and rubber (Vermeulen et al. 2005). However, some sulfur-containing compounds can contribute positive aromas to wine, such as strawberry, passion fruit, and grapefruit. The development of sulfur compounds is well understood in yeast, but less so in bacteria (Swiegers and Pretorius 2007).
The sulfur-containing amino acids methionine and cysteine can be metabolized by some LAB species. Methionine can be metabolized by *O. oeni* and *Lactobacillus* species to methanethiol, dimethyl sulfide, 3-(methylsulfanyl)propan-1-ol, and 3-(methylsulfuranyl)-propanoic acid (Pripis-Nicolau et al. 2004). Cysteine can be the precursor of S-containing heterocycles, such as thiazoles, and there is evidence that *O. oeni* is able to metabolize these compounds, resulting in aroma descriptors, including sulfury, floral, fruity, toasted, and roasted (Pripis-Nicolau et al. 2004).

### 17.3.3 N-Heterocyclic Compounds (Mousy Off-Flavor)

The production of N-heterocyclic compounds in wine results in an unpleasant odor that is reminiscent of mouse nests or mouse urine, and is often referred to as a mousy off-flavor. Interestingly, this aroma flavor can only be perceived on the back palate as a persistent aftertaste because of interactions of the wine with the mouth environment; an increase in pH renders the compounds volatile (Tucknott 1977). There are three sensorily important compounds responsible for the mousy aromas, 2-acetyltetrahydropyridine (ACTPY), 2-acetyl-1-pyrroline (ACPY), and 2-ethyltetrahydropyridine (ETPY) (Heresztyn 1986b).

Mousiness can be caused by *Dekkera/Brettanomyces* yeast, but mostly it is the LAB that are the major cause of this wine taint (Tucknott 1977; Grbin and Henschke 2000). The heterofermentative LAB, *O. oeni*, *Lc. mesenteroides*, and some *Lactobacillus* species are capable of synthesizing ACTPY, ACPY, and ETPY (Costello et al. 2001). The homofermentative LAB species, *Pediococcus* and *Lb. plantarum*, produce little or no detectable mousy compounds. The metabolism of lysine and ornithine in the presence of ethanol and fructose by *Lb. hilgardii* has been shown to lead to formation of the three compounds (Costello and Henschke 2002).

### 17.3.4 Malic Acid Metabolism

Malic acid metabolism by LAB is usually via the malolactic enzyme that constitutes MLF; l-malic acid is converted directly to l-lactic acid and CO$_2$ (Möslinger 1901). There are not many microorganisms that can utilize malic acid as a carbon source, and there are minimal reports on the presence of a malic enzyme or malate dehydrogenase in LAB. *Lb. casei* and *Lactococcus faealis* can covert malate to pyruvate with a malic enzyme that enables their growth on malate as a carbon source (Landete et al. 2010; London and Meyer 1969; London et al. 1971; Schütz and Radler 1974). *Lb. fermentum* has been demonstrated to produce d- and l-lactate, acetate, succinate, and CO$_2$ from malate (Radler 1986); at low pH (<pH 4), lactate production is favored, while at high pH (>pH 5) mainly succinate and acetate are formed. *Lb. delbrueckii* has been reported to possess a malate dehydrogenase (Caspritz et al. 1983; Lonvaud-Funel and Strasser de Saad 1982). All these

---

Table 17.1  Wine Spoilage Compounds Due to Bacterial Metabolism during Winemaking

<table>
<thead>
<tr>
<th>Compound</th>
<th>Origin or Metabolism</th>
<th>Sensory Descriptor</th>
<th>Aroma Threshold</th>
<th>Bacteria (Genus)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetic acid</td>
<td>Glucose and fructose, Citric acid</td>
<td>Vinegar, sour, pungent</td>
<td>0.2 g/l</td>
<td>LAB&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>Various, including sugar metabolism</td>
<td>Nail polish remover</td>
<td>7.5 mg/l</td>
<td>LAB</td>
</tr>
<tr>
<td>Diacetyl 2,3-butandione</td>
<td>Citric acid</td>
<td>Buttery, nutty, caramel</td>
<td>0.1–2 mg/l</td>
<td>Oenococcus Lactobacillus</td>
</tr>
<tr>
<td>2-Ethoxy-3,5-hexadiene</td>
<td>Sorbic acid</td>
<td>Crushed geranium leaves</td>
<td>0.1 µg/l</td>
<td>Lactobacillus Pediococcus</td>
</tr>
<tr>
<td>2-Acetyl-tetrahydropyridine</td>
<td>Fructose/glucose and lysine</td>
<td>Caged mouse</td>
<td>4–5 µg/l</td>
<td>Lactobacillus Oenococcus</td>
</tr>
<tr>
<td>(ACTPY)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-Ethyl-tetrahydropyridine</td>
<td>Unclear</td>
<td>Caged mouse</td>
<td>2–18 µg/l</td>
<td>Lactobacillus Oenococcus</td>
</tr>
<tr>
<td>(ETPY)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-Acetyl-1-pyrroline</td>
<td>Fructose/glucose and ornithine</td>
<td>Caged mouse</td>
<td>7–8 µg/l</td>
<td>Lactobacillus Oenococcus</td>
</tr>
<tr>
<td>(ACPY)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acrolein</td>
<td>Glycerol</td>
<td>Bitterness</td>
<td>10 mg/l</td>
<td>Lactobacillus Pediococcus</td>
</tr>
<tr>
<td>β-β-Glucan (exopolysaccharide)</td>
<td>Glucose</td>
<td>Ropy, viscous, oily, slimy, thick texture</td>
<td></td>
<td>Pediococcus</td>
</tr>
<tr>
<td>Mannitol</td>
<td>Fructose</td>
<td>Viscous, sweet, irritating finish</td>
<td></td>
<td>Oenococcus</td>
</tr>
<tr>
<td>Histamine</td>
<td>Decarboxylation of histidine</td>
<td>Physiological reactions</td>
<td></td>
<td>LAB</td>
</tr>
<tr>
<td>Biogenic amines</td>
<td>Mainly decarboxylation of amino acid</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Volatile Phenols 4-Ethylphenol</td>
<td>p-Coumaric acid</td>
<td>Medicinal, barnyard</td>
<td>0.14–0.6 mg/l</td>
<td>Lactobacillus</td>
</tr>
<tr>
<td>Sulfur Compounds Methanethiol</td>
<td>Methionine</td>
<td>Cooked cabbage, rubber</td>
<td>0.3 µg/l</td>
<td>Oenococcus (potentially)</td>
</tr>
</tbody>
</table>

(continued)
alternative malate metabolisms would be considered spoilage as most of the secondary metabolites produced are undesirable in wine.

### 17.3.5 Citric Acid Metabolism

Citric acid will be metabolized by many LAB during their growth in red and white wines. Numerous secondary metabolites are produced, of which acetic acid and diacetyl (2,3-butanediol) have the greatest sensory impact. Both compounds will contribute complexity to wine at lower concentrations and at higher concentrations will be considered spoilage. Citric metabolism commences after approximately 60–75% of malic acid has been metabolized and is usually completed 1 week after MLF is deemed complete (Bartowsky and Henschke 2004; Krieger et al. 2000).

The first step of citric acid metabolism results in the release of acetic acid to oxaloacetic acid and then onto pyruvic acid, where under acidic conditions, the metabolism favors the production of diacetyl and the final point of the pathway, 2,3-butanediol (Ramos and Santos 1996). There is always a small increase in volatile acidity (acetic acid) in wine following MLF, due to citric acid metabolism; however, this is not the main contributor to undesirable quantities of acetic acid. Elevated concentrations of acetic acid, 0.7–1.1 g/l and higher, impart a vinegar-like character to wine (Corison et al. 1979), and can be due to metabolism of AAB species (Bartowsky et al. 2003; Bartowsky and Henschke 2008), yeast metabolism (Swiegers et al. 2005), or metabolism of sugars by LAB, including *Lb. kunkeei* (Edwards et al. 1998; Edwards et al. 1999).

Diacetyl is an important bacterial secondary metabolite in wine. This compound imparts a buttery, nutty, or butterscotch aroma to wine, and desirability is concentration dependent; up to ~4 mg/l, it contributes to wine complexity, but over 7 mg/l it is considered objectionable (Martineau et al. 1995; Rankine et al. 1969). The sensory perception of diacetyl is also dependent on the wine matrix, age, style, and origin of the wine (Martineau and Henick-Kling 1995; Bartowsky et al. 2002). The concentration of diacetyl can be relatively easily manipulated during the winemaking process to achieve desired sensory attributes (Bartowsky and Henschke 2004).

### 17.3.6 Tartaric Acid Metabolism

The capacity to metabolize one of the most important organic acids in wine, tartaric acid, is a rare bacterial characteristic. Even though Pasteur described the metabolism of tartaric acid by LAB,
and named it “tourne” disease, few reports exist on this wine spoilage. Only two Lactobacillus species (Lb. plantarum and Lb. brevis) have been demonstrated to metabolize tartaric acid to acetic acid, succinic acid, and CO₂ (Radler et al. 1972; Sponholz 1993; Ribéreau-Gayon et al. 2006). The main enzyme, tartrate dehydratase, is inducible in both Lactobacillus species, but with slightly different metabolic pathways. The anaerobic metabolism by homofermentative Lb. plantarum from 1 mol of tartrate is 1.5 mol CO₂, 0.5 mol acetic acid, and 0.5 mol lactic acid, and from the heterofermentative Lb. brevis, 1.33 CO₂, 0.67 mol of acetic acid, and 0.3 succinic acid are formed (Radler and Yannissi 1972). Wines demonstrating this spoilage are often described as fizzy, limp, flat, dull, and cloudy. Management of these bacteria is usually by maintaining low wine pH (higher acidity) and a good SO₂ regime.

17.3.7 Lactic Fermentation of Glycerol (Acrolein)

The metabolism of glycerol by LAB, particularly in red wine, can result in bitterness, often referred to as acrolein taint. Again, this is a well-known spoilage disease already described by Pasteur (1873), who connected the presence of rod-shaped bacteria with the loss of glycerol, and Voisenet (1910, 1911) linked this with bitterness of red wine. Acrolein itself is not bitter, but is reacts with the phenolics groups of anthocyanins to produce a bitter sensation (Rentschler and Tanner 1951); hence it is associated more with red than white wines. Sensory threshold concentrations are not clear; however, acrolein concentrations as low as 10 mg/l have been shown to cause a bitter taint (Margalith 1983).

The key enzyme in anaerobic glycerol metabolism is glycerol dehydratase converting glycerol to 3-hydroxypropionaldehyde with acrolein spontaneously forming following exposure to heat or long-term storage in acidic solutions (such as wine) (Schütz and Radler 1984; Smiley and Sobolov 1962). The genes for this pathway have been studied in Lb. collinoides, Lb. hilgardii, and Lb. dio-livorans and are organized in an operon of 13 genes, most likely all necessary for the functioning of the three protein subunits of glycerol dehydratase and propane-1,3-diol-dehydrogenase (Gorga et al. 2002). A second pathway for the degradation of glycerol, oxidative branch, uses 3-P-glycerol dehydrogenase resulting in 3-P-dehydroxyacetone, which enters into glycolysis reactions and results in a suite of compounds, including lactic acid, acetic acid, and acetoinic compounds (Bauer et al. 2010a; Sobolov and Smiley 1960).

Lactobacillus species appear to be the only LAB linked to glycerol metabolism and acrolein formation (Pasteris and Strasser de Saad 2009; Bauer et al. 2010b; Garai-Ibabe et al. 2008; Sauvageot et al. 2000; Martin et al. 2005). P. pentosaceus can aerobically metabolize glycerol to undesirable flavor compounds, but acrolein is not one of these (Pasteris and Strasser de Saad 2005). In a study of wine-associated LAB, it was demonstrated that the ability to ferment glycerol is limited (1% O. oeni, 12% P. parvulus, and 31% Lactobacillus sp.); however, the assay was conducted under aerobic conditions and does not infer the ability to produce acrolein (Davis et al. 1988). Several other bacteria are able to transform glycerol to 3-hydroxypropionaldehyde anaerobically; Bacillus, Klebsiella, Citrobacter, Enterobacter, and Clostridium (Bauer et al. 2010a; Vollenweider and Lacroix 2004).

Management of these LAB in wine to minimize the fermentation of glycerol to produce acrolein mainly revolve on maintaining low pH and higher SO₂ regime to minimize their growth (Ribéreau-Gayon et al. 2006).

17.3.8 Sorbic Acid Metabolism (Geranium Off-Flavor)

Sorbic acid (2,4-hexadienoic acid) can be used as a chemical preservative in sweet wines at bottling to prevent yeast fermentation after packaging. At concentrations used in wine (200 mg/l), its
antimicrobial activity is ineffective against LAB (Edinger and Splittstoesser 1986a, 1986b). Sorbic acid can be metabolized by LAB species, including *O. oeni* to 2-ethoxyhexa-3,5-diene, which has an odor reminiscent of crushed geranium leaves (*Pelargonium* spp.) (Riesen 1992; Crowell and Guymon 1975; Edinger et al. 1986a, 1986b). A small amount of ethanol is required, thus this spoilage is not observed in grape juice; however, it can occur if grape juice is added to wine, to sweeten it (Sponholz 1993).

### 17.3.9 Mannitol (Fructose Metabolism)

Mannite disease was first described by Pasteur (1873) and is due to fructose reduction by both heterofermentative and homofermentative bacteria resulting in the formation of mannitol, a six-carbon sugar alcohol (polyol), which is perceived as sliminess with a vinegary–estery, slightly sweet taste in wine (von Weymarn et al. 2002; Wisselink et al. 2002). In general, the homofermentative LAB will only produce small amounts of mannitol, whereas some heterofermentative LAB produce substantial amounts of mannitol (Wisselink et al. 2002). The heterofermentative *Lb. brevis* has been shown to produce significant amounts of mannitol from fructose (Martinez et al. 1963). In *O. oeni*, fructose can be metabolized by two different pathways: heterolactic fermentation or mixed heterolactic/mannitol fermentation (Richter et al. 2003). The switch from one fermentation type to the other occurs at the metabolic level and is related to the growth rate.

This type of wine spoilage tends to be complex, as it is also accompanied by high concentrations of acetic acid, D-lactic acid, propanol, 2-butanol, and diacetyl (Sponholz 1993).

### 17.3.10 Exopolysaccharide Metabolism

The production of exopolysaccharides is almost exclusively due to *Pediococcus* growth and metabolism of glucose in wine, usually *P. parvulus* (Werning et al. 2006; Dols-Lafargue et al. 2008). Wines spoiled due to exopolysaccharide production are referred to as having “ropy,” or “graisse” disease (as described by Pasteur) and are viscous, slimy, oily, and have a thick texture. Viscosity of wine can add mouthfeel and be a positive feature, although not at the concentrations when produced through the growth of spoilage LAB, particularly *Pediococcus* species. It has been observed that increases in wine viscosity occur when both LAB and AAB are present (Lüthi 1957).

The presence of residual sugar (glucose) in wine, even at low concentrations (<50 mg/l), with the appropriate growth conditions can allow for the formation of exopolysaccharides or β-glucan in wine. Glucan production has been shown to be greater in nutrient-poor medium; more glucan is produced in medium containing 0.1 g/l glucose than 2 g/l (Walling et al. 2005a). Where nitrogen is limiting in the same glucose concentration, the production of glucan by *P. parvulus* will be greater. Thus wines with a high pH, low glucose, and nitrogen concentrations and no agitation are likely to become ropy (Walling et al. 2005a).

In wine, this exopolysaccharide is a high-molecular-weight β-glucan, a glucose homopolymer that consists of a trisaccharide repeating unit with a β-1,3-linked D-glucosyl backbone and branches made up of single β-1,2-linked D-glucopyranosyl residues (Llauberes et al. 1990; Duenas-Chasco et al. 1998; Duenas-Chasco et al. 1997). This type of glucan cannot be removed by enzymatic treatment with currently known enzymes. The pathway for the production β-1-glucan and the polymerization is well characterized (Walling et al. 2005a).

The presence of a plasmid carrying the *dps* gene (glucan synthase responsible for the polymerization of glucan residues) is required for *Pediococcus* production of β-1-glucan and ropy wines (Walling et al. 2005b). Recent studies have shown that some strains of *O. oeni* carry the *dps* gene (Walling et al. 2005b). The glucosyltransferase gene, *gtf*, has been characterized in *P. parvulus* and
O. oeni (Dols-Lafargue et al. 2008). Recent studies have demonstrated that O. oeni strains can produce exopolysaccharides, although usually in much lower concentrations as found in spoiled ropy wines (Ciezack et al. 2010).

17.3.11 Biogenic Amine Production

Biogenic amines are organic nitrogenous bases of low molecular weight that are formed during the metabolism of living organisms (Smit et al. 2008). They are found in a range of fermented foods and beverages, including wine. When absorbed at a too high concentration, biogenic amines can have undesirable physiological effects, including headaches, as well as gastrointestinal and respiratory distress.

The principal biogenic amines found in wine are histamine, tyramine, putrescine, cadaverine, phenylethylamine, spermidine, spermine, agamatine, and tryptamine. The main microbial source of these compounds in wine is LAB (Coton et al. 1998; Lonvaud-Funel and Joyeux 1994; Soufleros et al. 1998; Lonvaud-Funel 2001). It has been established that red wine generally exhibits higher biogenic amine concentrations than that of white wine, which is partly attributed to the greater susceptibility of red wine to undergo MLF (Bartowsky and Stockley 2011; Vidal-Carou et al. 1990). Biogenic amines in wine have been reviewed recently by several research groups (Smit et al. 2008; Ancin-Azpilicueta et al. 2008; Anli and Bayram 2009; Ferreira et al. 2006).

Histamine is the main biogenic amine in wine and is the most associated with negative health implications. It is formed by the decarboxylation of the amino acid l-histidine and the International Organization of Vine and Wine has proposed a histamine limit of 10 mg/l in red and white wines (Anli and Bayram 2009). The histidine decarboxylase enzyme has been purified and characterized. The protein is a single polypeptide of 315 amino acids, comprising two subunits, α and β, forming a hexamer (Lonvaud-Funel and Joyeux 1994). The decarboxylation reaction does not directly generate energy; however, the exchange of histidine and histamine at the membrane level creates a proton gradient and a proton motive force, generating ATP (Rollan et al. 1995). In Lb. hilgardii, the histidine decarboxylase activity was associated with the presence of an 80-kb plasmid; four genes in a cluster: hdcP (histidine/histamine exchanger), hdcA (structural protein), hdcRS (histidyl-tRNA synthetase), and hdcB (unknown product) (Lucas et al. 2005). There is evidence that the same gene cluster is present in other LAB, including Lactobacillus 30a and O. oeni 9204 (Gevers et al. 2003), and the plasmid-encoded system could be transferred horizontally (Lucas et al. 2005). The ability of wine LAB species to produce histamine varies with both species and strain. Strains of Lactobacillus, Pediococcus, Leuconostoc, and O. oeni have been demonstrated to produce histamine (Landete et al. 2005; Marcobal et al. 2006).

Tyramine is formed by the direct decarboxylation of tyrosine with four well-characterized genes (tyrosyl-tRNA synthetase, tyrosine decarboxylase, probable tyrosine permease, and Na+/H+ antiporter) (Lucas and Lonvaud-Funel 2002; Lucas et al. 2003). Tyramine does not appear to be produced by O. oeni strains (Moreno-Arribas et al. 2000; Guerrini et al. 2002).

Putrescine is reported to be the most abundant biogenic amine in wine, both qualitatively and quantitatively (Smit et al. 2008; Bartowsky and Stockley 2011). Ornithine is decarboxylated by ornithine decarboxylase (ODC) to putrescine, with the ODC gene first identified in O. oeni (Marcobal et al. 2004; Marcobal et al. 2005). Even though there can often be high concentrations of putrescine present in wines following MLF (Gloria et al. 1998; Bartowsky and Stockley 2010), the presence of the ODC gene does not appear to be widespread (Marcobal et al. 2004).

An alternative pathway for the production of putrescine has been proposed from arginine (Mangani et al. 2005). This might explain the high concentrations of putrescine in wine, and the
minimal presence of the ODC gene in wine LAB. With this pathway, arginine can be catabolized via arginine deiminase (ADI), ornithine transcarbamoylase, and carbamate kinase (Mangani et al. 2005; Smit et al. 2008). This pathway has also been demonstrated in *Lb. hilgardii* (Arena et al. 2001).

### 17.3.12 Indole Production

A high concentration of indole has been linked to “plastic-like” off aromas, predominantly in wines produced under sluggish fermentation conditions (Capone et al. 2010). Indole has an aroma detection threshold of 23 µg/l in white wine (Capone et al. 2010), and its formation is wine is not understood, but is likely to be related to tryptophan metabolism. Tryptophan is an aromatic amino acid and its metabolism has been linked to other off-flavors such as “untypical aging off-flavor” in wine (Hoenicke et al. 2002). Catabolism of tryptophan by different microorganisms has been observed, including *Lb. casei* and *Lb. helveticus*, which can form aromatic compounds, including indole, which impart putrid, fecal, and unclean flavors to cheese (Gummalla and Broadbent 1999). Strains of *Lb. lindneri*, *P. parvulus*, *P. cerevisiae*, and *O. oeni* have been demonstrated to generate indole during MLF, and this ability was dependent on the presence of tryptophan in the medium (Arevalo-Villena et al. 2010).

### 17.3.13 Ethyl Carbamate

Ethyl carbamate, also referred to as urethane, is genotoxic and carcinogenic to animals, and is formed through the chemical reaction of ethanol and citrulline, urea, or carbamyl phosphate. Arginine, a quantitatively important amino acid of grape must and wine (Ough et al. 1988; Henschke and Jiranek 1993), is a precursor of citrulline, and LAB vary in their ability to degrade arginine (Granchi et al. 1998).

*O. oeni* and *Lb. buchneri* are able to metabolize arginine and citrulline (Mira de Orduna et al. 2000). Typical wine parameters (high ethanol, high L-malic acid, low pH) are conducive to ethyl carbamate production; however, concentrations will increase even more at higher pH (Romero et al. 2009). The presence of the *arc* genes, for the arginine–deiminase pathway, has been identified in several genera of wine LAB. A correlation was found between the presence of genes and the ability to degrade arginine; degrading strains included all heterofermentative lactobacilli, *O. oeni*, *P. pentosaceus*, and some strains of *Lc. mesenteroides* and *Lb. plantarum* (Araque et al. 2009).

The role that MLF plays in the potential ethyl carbamate remains unclear; however, experiments conducted in synthetic wine and a laboratory vinified wine demonstrated a correlation between arginine degradation, citrulline production, and the ethyl carbamate formation. Even though the formation of this compound does not necessarily affect the wine sensory quality, it can potentially have health implications (Weber and Sharypov 2009).

### 17.4 Conclusions—Summary

The best wine is achieved by maximizing the desired aromas and flavors and minimizing the less desirable off-flavors and off-aromas. This chapter has sought to summarize the bacterial and chemical interactions that contribute to undesirable or spoilage wine aromas and flavors. Often it is a fine balance between the concentration of a secondary metabolite, it being considered desirable and moving onto spoilage. Wine-associated LAB cannot always be easily divided into effective or
contaminating bacteria. Managing the bacterial flora of fermenting grape juice and maturing wine is not always an easy task for the winemaker. Wine acidity (pH) management and timing of sulfur regimens are the most common practices to control contaminant LAB. This chapter did not explore ways to control wine-contaminating LAB; however, there are a few emerging technologies, including UV irradiation, pressure, and electric fields that have been employed in numerous beverage industries and are beginning to be explored in the wine industry (reviewed by Bartowsky 2009).

Acknowledgments

The Australian Wine Research Institute, a member of the Wine Innovation Cluster in Adelaide, is supported by Australia’s grapegrowers and winemakers through their investment agency, the Grape and Wine Research and Development Corporation, with matching funds from the Australian government.

References


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Chapter 18

Stability of Lactic Acid Bacteria in Foods and Supplements

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18.1 Introduction

Lactic acid bacteria (LAB) have been part of human nutrition since ancient times. These microorganisms were initially responsible for spontaneous food fermentations, which provided a vast array of fermented products. With the intention of controlling these spontaneous and initially uncontrolled processes, specific LAB were propagated and used in what, very likely, constituted the origin of food microbiology. More recently, some LAB as well as bifidobacteria have started to be extensively used owing to their health-promoting properties; these are the so-called probiotics. Increasing interest has arisen in recent years in relation to the inclusion of probiotic strains in foods and supplements, and a great variety of products have been formulated worldwide. An accurate enumeration and viability assessment of these microorganisms in such products is of great importance to both manufacturers and consumers. The most important quality criteria for probiotic-containing products are the identity of the strains used and the level of viable cells in the product.

In general, LAB used as starters to ferment foods are tolerant to the conditions generated during fermentation; otherwise, they could not complete the fermentative process. Usually these strains show good stability in the final product. However, some LAB and *Bifidobacterium* strains used as adjunct cultures for probiotic applications are highly sensitive to environmental conditions, showing poor survival, which constitutes one of the main problems that manufacturers have to face. The stability of probiotics must be high enough, during the whole shelf life of the product, to guarantee the daily dose needed to provide the health benefits attributed to the specific product or preparation. Some probiotics and LAB strains show poor stability in market preparations with many different factors, such as pH, acidity, presence of other microorganisms, temperature and oxygen content, among others, affecting survival and viability of the strains (Dave and Shah 1997; Ravula and Shah 1998; Gueimonde et al. 2004b). In addition, not only the stability of the strains in terms of survival, but also in terms of metabolic activity, is needed to maintain the desired sensory attributes during the whole shelf life of the product. Although not extensively studied yet, the introduction of probiotics in foods and supplements also requires stability of the probiotic properties of the strains, to ensure that the consumer gets the expected benefit from the product.

Moreover, stability in the product might not be enough and it is also important that, following consumption, probiotics remain viable at sufficient levels during the gastrointestinal tract (GIT) passage. After ingestion, these cultures must overcome different biological barriers. In this regard, the strains should be stable when exposed to the harsh conditions of the human intestinal tract, including enzymes, acidic pH, and bile salts, to be able to reach their site of action and still be able to exert their beneficial effect.

In spite of the availability of culture-independent molecular tools for stability assessment, most manufacturers rely on conventional culture techniques, as also legislators require. The current availability of faster and more accurate methods, which provide complementary information to the traditional ones, promises to expand our understanding of the stability of LAB and probiotics in foods and supplements, as well as during GIT transit.

In this chapter we aim to describe the factors affecting the stability of LAB and probiotics in both foods and supplements, and some potential strategies to improve it, as well as the different methods currently available to measure the viability of LAB and probiotics.

18.2 Factors Affecting Stability

When a microorganism is intended to be used during food manufacture, it has to be able to cope with the many different stresses present throughout both the processing and storage of the
product. Among these stresses affecting the stability of microorganisms, the principal industrial factors are low and high temperatures, water activity ($a_w$), oxygen, acidity, and the presence of chemicals or other microorganisms that may inhibit the strain(s) being used. These factors, starting from the strain production process till the storage conditions of the final product, may have a profound effect on the survival, stability, and properties of LAB and probiotics. Therefore, precise knowledge of the manufacturing conditions and understanding of how the processes may affect the microorganisms being used are needed for the development of a successful product containing stable LAB and/or bifidobacteria (Figure 18.1).

**18.2.1 Strain and Strain Production Conditions**

Resistance to the technological production processes is of paramount importance to obtain an effective biomass production. It is well known that some probiotic and LAB isolates are fastidious microorganisms that grow poorly in industrial media. The impossibility of growing a strain in high numbers may impede its commercialization, as often high bacterial counts are needed in the final product (Lee and Salminen 1995). Therefore, several parameters on strain production have to be taken into account, among which optimization of growth and processing conditions, and monitoring of loss of viability during manufacturing and storage are of key importance (Ananta et al. 2005).

The effectiveness of large-scale cultivation seems to be very dependent on the strain’s own intrinsic tolerance to stress. Thus, this is the first factor that should be taken into account. Different strains may show large differences in their ability to cope with different manufacturing conditions. Therefore, the identification of those strains showing higher tolerance, or improvement in the properties of the strain, constitute one of the primary targets. Several strategies are under investigation to improve delivery, specificity, and efficacy, by ameliorating strain properties, among them the principal are stress adaptation and gene modification. Exposure of the strains to a sublethal stress has been shown to increase the survival of the microorganisms during product manufacture and storage (Maus and Ingham 2003; Saarela et al. 2004).

Resistant derivatives are relatively easy to obtain by exposing sensitive strains to stressing environmental factors (oxygen, heat, freezing, drying, acid, bile salts, NaCl, etc.). Resistant microorganisms usually present a stable stress-resistant phenotype, with changes affecting not only the targeted property but often also presenting cross-resistance to other stresses. The use of these
stress-resistant strains can be useful for improving viability during food processing and afterward during gastrointestinal transit. However, care should be taken as the stress adaptation may alter other physiological and functional properties of the strain (Noriega et al. 2004; Guglielmotti et al. 2007; Sánchez et al. 2008). For instance, acquisition of resistance to bile salt has been shown to increase resistance to low pH, inducing stable changes in carbohydrate fermentation and glycosidase activities (Noriega et al. 2004). This acquisition induced further changes and affected the ability of the strains to adhere to human intestinal mucus (Gueimonde et al. 2005), which correlated with an increase in the ability to inhibit the adhesion of pathogens to human mucus (Gueimonde et al. 2007).

An alternative approach is to enhance the stress tolerance of the strains by gene modification. Strains may be genetically modified to accumulate protective compounds (compatible solutes) to improve stress tolerance. This strategy was used to increase salt, chill, and cryotolerance of a Lactobacillus strain (Sheehan et al. 2006), which additionally improved the freeze- and spray-drying survival of the strain. This approach was also used to increase Bifidobacterium breve (Sheehan et al. 2007a) and Lactococcus lactis (Termont et al. 2006) tolerance to acidic environments.

In addition to the intrinsic tolerance to stress conditions, the industrial strain production processes may themselves also have a deep impact on the stability of the strains. Among these, strain freezing and drying conditions, as well as the matrix characteristics, such as $a_w$ of the product or oxygen concentration, will determine, to a great extent, the strain stability.

### 18.2.1.1 Freezing

Strains for food applications are often supplied as frozen cultures. In the case of dairy products, adding strains as concentrated frozen starters to milk presents the advantage of allowing a direct inoculation of vats.

Before freezing, bacteria are usually grown in synthetic or dairy-based media (when possible) until early stationary phase of growth, usually being concentrated by centrifugation (Holzapfel et al. 2001). They are resuspended afterward in a suitable culture medium that includes a cryoprotectant to protect bacterial cells from damages due to ice crystal formation. To date, several ways of obtaining frozen concentrates of probiotics, or LAB, have been developed, using different cryoprotectants (Ross et al. 2005). The selection of the most appropriate one plays a key role in the further stability of the strain during the freezing process. However, the use of freezing is nowadays being replaced by drying techniques, which are more cost effective and less time consuming (Ross et al. 2005).

### 18.2.1.2 Drying

Drying, which is simply water elimination from the bacterial suspension, involves strain dehydration through two main techniques, freeze-drying and spray-drying. To date, drying has been extensively used for the production of bacteria included in foods and probiotic products (Ananta et al. 2005). In general, drying of cultures renders high production rates, and dehydrated powders are stable and easy to store and transport.

One of the techniques most commonly used is spray-drying, which comprises the atomization of the bacterial suspension into a drying gas, thus resulting in fast water evaporation (Picot and Lacroix 2004). Spray-drying involves the use of a carrier molecule, such as soluble starch, arabic gum, skimmed milk, gelatin, or polysaccharides, among others (Lian et al. 2002, 2003; Hsiao et al. 2004). Spray-drying is an effective technology, but has the disadvantage of needing high
temperatures for water evaporation during the passage of bacteria through the drying chamber, which also promotes a relatively harsh osmotic stress (Teixeira et al. 1995; Gardiner et al. 2002). This results in simultaneous dehydration, thermal and oxygen stresses that are imposed on the microorganisms, leading to a reduction of both viability and activity (Anal and Singh 2007). Several scientific works have reported on the use of protectants, such as trehalose, nonfat milk solids, growth-promoting factors, and prebiotics, to increase stability in dry form (Crittenden et al. 2001; Corcoran et al. 2004). In this way, it has been observed that some bacterial strains can be spray-dried without suffering dramatic decreases in their viability (Silva et al. 2002). For these reasons, spray-drying is considered, when possible, a convenient and cost-effective technique for the large-scale preparation of bacterial powders.

Freeze-drying is known as one of the most convenient methods for drying biological and sensitive materials. In the case of LAB and bifidobacteria, this process has been considered for a long time a proper method of obtaining a stable formulation. However, leakage of cellular content due to cytoplasmic membrane injuries has been observed in some cases (Teixeira et al. 1996; Castro et al. 1997). This has been tried to be solved by the addition of cryoprotectants, which have been extensively used for increasing bacterial viability (Ross et al. 2005). Choosing an appropriate drying medium is thus of paramount importance. For several LAB and probiotic bacteria, skimmed milk powder is the drying medium of choice, since it has been shown to prevent cellular injuries by stabilizing the cytoplasmic membrane. Skimmed milk powder also facilitates, notably, the rehydration of the dried preparation, and it provides a protective layer that coats cells (Abadias et al. 2001).

Spray-drying and freeze-drying can be combined to obtain powders in the so-called “spray-freeze-drying.” This technique combines the extrusion device from the spray-dryers followed by a freeze-drying module, which yields a dry powder with a narrow particle size distribution (Semyonov et al. 2010). Spray-freeze-dried powders have the advantage of having a controlled particle size and a larger specific surface area; these characteristics allow, for instance, further coating with food-grade polymers (Yu et al. 2006).

Finally, it has to be highlighted that due to the high intra- and interspecies variability, the optimum cryoprotectant, or carrier molecule, and its appropriate concentration during and following drying, has to be chosen on a case-by-case basis (Carvalho et al. 2004). In addition, growth, drying, storage, and rehydration conditions have to be extensively optimized to maximize the recovery and stability of dried cultures.

18.2.2 Matrix and Food Manufacturing Processes

As already mentioned, some bifidobacteria and LAB strains may beneficially affect human health through different mechanisms. Quite often, the number of probiotic bacteria ingested in a functional food is low, which is compromising for the claimed benefit. Certain factors in the manufacturing processes have a negative impact on bacterial viability, thus conditioning the survival of the strains in both food and GIT. Several approaches, such as microencapsulation, have been applied to enhance the viability of the strains in food matrices (Rokka and Rantamaki 2010).

Among these food matrix and manufacturing factors, \( a_w \) and oxygen concentration are key factors negatively affecting probiotic survival, especially when these are included in nondairy products (Mattila-Sandholm et al. 2002). Keeping \( a_w \) at low values is a benefit from the point of view of the strain stability. Nevertheless, these low \( a_w \) values can be reached in dry products, capsules, or supplements but they are not often present in food products. It has also been suggested that exopolysaccharide-producing strains may somehow be protected more from these stresses (Roberts et al. 1995).
18.2.2.1 Water Activity

When applied to food matrices, \( a_w \) is defined as the quotient of vapor pressure of water in food and the vapor pressure of pure water. Thus, water \( a_w \) is a way of measuring the free moisture in foods; \( a_w \) is thus something different from water content. \( a_w \) values range from 0 to 1, 1 being the value of water activity corresponding to pure water.

Probiotics and LAB are usually added to fresh foods with high \( a_w \), such as milk, fermented milks or juices, which have an expected shelf life of weeks. However, probiotics can also be added to dry products with low \( a_w \) (\( a_w < 0.25 \)), which in turn have an expected shelf life of months (e.g., infant formula). In this way, \( a_w \) has a critical effect during the long-term storage of microorganisms (Abe et al. 2009a).

In the case of dried strains, \( a_w \) can vary from <0.03 (freeze-dried bacterial powders) to 0.3 (spray-dried emulsions) (Crittenden et al. 2006; Saarela et al. 2006). In spray-drying, \( a_w \) is directly dependent on the temperature, and thus, the lower the outlet temperature is, the higher the moisture of the dried powder (Gardiner et al. 2002; Ananta et al. 2005). Product shelf life is highly dependent on \( a_w \); it is generally accepted that viability is negatively affected when the dried matrix has a value higher than 0.25 (Teixeira et al. 1995).

18.2.2.2 Oxygen Concentration

Tolerance to oxygen is a useful trait for the successful incorporation of probiotic bacteria, such as bifidobacteria, into food (Kullisaar et al. 2002). Probiotics are being included in a high diversity of food-grade matrices, ranging from fermented milks to dietary supplements. With the exception of functional foods and supplements packed under controlled atmospheres, oxygen is one of the abiotic factors negatively affecting the survival of probiotics (Kullisaar et al. 2002). The toxic effect of oxygen is mainly due to induction of oxidative damage.

Oxidative damage is basically induced by the presence of the so-called reactive oxygen species (ROS). Bacteria have developed several mechanisms for counteracting these oxidative effects, among which glutathione production and antioxidative enzymes such as catalase or superoxide dismutase are worth mentioning (Halliwell et al. 1992; Miller and Britigan 1997). Probiotic bacteria, which are mainly members of the Actinobacteria and Firmicutes divisions, counteract the toxic effects of ROS with the production of flavoproteins, which, in turn, results in the production of compounds with high antibacterial activity, such as hydrogen peroxide (Kullisaar et al. 2002). It is known that certain LAB can decrease the ROS accumulation in foods, for this reason they are considered antioxidant microorganisms (Korpela et al. 1997). This trait seems to be related to the production of cytoplasmic-associated superoxide dismutase activity, and with an enhanced activity to reduce the hydroxyl radicals in cell suspensions.

Aerobic conditions are present during the manufacturing process and storage of functional foods, aerotolerance thus being a desired trait for strains used in industry. Aerotolerance shows a high strain-to-strain variation and, in some cases, a single species is the most commonly used in the industry just due to its good resistance to oxidative and other stresses (for instance the case of Bifidobacterium animalis subsp. lactis).

18.2.2.3 Chemical and Microbiological Composition

Several factors relating to the matrix’s chemical and microbiological composition are involved in affecting the stability of probiotics and LAB cultures in foods and, therefore, should be taken into
account when formulating a product. Most of the studies within this field have been carried out with dairy applications and are difficult to extrapolate to other products.

With regard to chemical composition, the presence of antimicrobial compounds in the raw material may affect the starter culture activity, which may lead to a failure in the fermentative process. The pH of the product is one of the main limiting factors affecting strain survival. In this regard, preadaptation of strains to low pH has been reported to improve subsequent survival (Saarela et al. 2009). Moreover, the chemical composition of the raw material used in the elaboration process, such as the fat content, may have an impact on strain survival (Vinderola et al. 2000a). It has also been reported that the use of food additives, such as flavorings or colorants, may negatively affect the stability of certain starter and probiotic strains (Vinderola et al. 2002a). In contrast, different carbohydrates such as fructooligossacharides, inulin, or β-glucan, as well as protein concentrates, have been used to improve the stability of probiotics in foods (Akalin et al. 2007; Donkor et al. 2007; Vasiljevic et al. 2007; Oliveira et al. 2009). Furthermore, the addition of prebiotics may favor the production of health-promoting metabolites (Oliveira et al. 2009).

Also, interactions among the strains included in the specific combination of microorganisms to be used need to be evaluated. Although studies in this area are still scarce, inhibitory activity of strains of *Lactobacillus delbrueckii* ssp. *bulgaricus* against strains of the other yogurt microorganism, *Streptococcus thermophilus*, has been reported. Similarly, interactions were also observed among other LAB and probiotic strains (Sodini et al. 2002; Vinderola et al. 2002b). It is also well known that some LAB are able to produce bacteriocins, which may inhibit the growth of closely related microorganisms. Thus, it is important to assess the properties and compatibility among strains used in combination. However, the effect of the presence of other microorganisms is not always detrimental. Fermentation at a low temperature (37°C) together with the presence of *Lc. lactis* in the starter, has recently been found to improve *B. longum* survival in yogurt (Abe et al. 2009c) and the combined use of yeasts, together with the bacteria, may also increase the survival of the bacterial strain (Liu and Tsao 2009).

It is important to underline that these effects appear to be dependent on the specific compounds and strains being used and, therefore, a careful case-by-case assessment of the different combinations is always needed.

### 18.2.3 Storage

Apart from trying to improve the strains and industrial processes, the packaging and storage can also be technologically modified, to favor the survival of the microorganism in the product or, later on, in the intestinal ecosystem. In this regard storage temperature and oxygen content in the matrix/package are known to play a key role in strain survival. Storage under refrigeration temperatures is known to enhance the survival of the strains in foods (Rozada et al. 2009). Storage of fermented products has often been related with a phenomenon called postacidification, which has been repeatedly reported to adversely affect the stability of probiotics in these products. Postacidification is caused by the production of acid by the starter strains during refrigerated storage of the product. An option to increase stability may consist of the use of strains lacking the ability to postacidify (Ongol et al. 2007).

As stated above, the oxygen content may also have an influence. Therefore, reducing the oxygen permeation of the product or reducing its redox potential by adding oxygen-scavenging agents have been strategies assessed as a means of improving strain stability (Shah 2000). In recent years, developments in the field of active packaging and modified atmospheres have also provided means for enhancing survival of the strains during storage of the product. Packaging under modified atmospheres allows a reduced oxygen content, which would benefit the survival of the strain in
the product. Also, carbon dioxide addition to liquid matrices, such as milk, which would partially displace the oxygen present, may have applications in this field (Gueimonde and de los Reyes-Gavilán 2004; Hotchkiss et al. 2006). For instance, the addition of carbon dioxide to milk has been shown to be a suitable option in yogurt manufacture (Gueimonde et al. 2003). This approach was also found to be appropriate for other probiotic-containing fermented milks (Vinderola et al. 2000b; Gueimonde et al. 2002). In addition, this process may inhibit the growth of deleterious microorganisms contributing to the reduction of the risk of contamination (Noriega et al. 2003).

Encapsulation of probiotics in different matrices was also found to improve the tolerance of the encapsulated strains to stressful environmental conditions such as those found during the gastrointestinal transit (Fávaro-Trindade and Grosso 2002; Dink and Shah 2009).

18.3 Stability in Foods and Supplements

Probiotics are currently included in a wide variety of formulations, such as fermented milk, chewing gum, capsules, or tablets, among others. Generally, a progressive loss of stability is observed during storage, which has raised the need for developing formulations that protect bacteria from harsh technological and environmental conditions (Klayraung et al. 2009). To exert their potential health-promoting effects, probiotic bacteria must survive in sufficient numbers in the probiotic product, if such a product aims to be marketable. In addition, their stability during product storage must be guaranteed, which includes all the properties by which a given bacteria is claimed to be probiotic. For example, the existence of potential negative interactions must be assessed between the strains and the food. Thus, it must be shown that probiotic cells survive in adequate levels throughout the shelf life of the functional food. This is a basic requirement to ensure that probiotic bacteria remain active during the shelf life of the product.

18.3.1 Stability in Dairy Products

Traditionally, LAB acting as starters or contributing to the ripening in fermented food products mainly belong to genera *Lactococcus*, *Leuconostoc*, *Pediococcus*, *Streptococcus*, and *Lactobacillus*, and they normally display appropriate stability in food matrices. When this is not the case, failure in acidification and maturation processes normally occurs. Foods containing probiotic pediococci, enterococci, lactobacilli, and bifidobacteria are currently well established in the market, with fermented dairy products, and particularly fermented milks, being, by far, the most important vehicles of probiotic administration to humans. To provide health benefits, a minimum concentration of viable probiotics in the product at the end of the refrigerated storage is suggested to be $10^6$–$10^7$ cfu/ml or gram (IDF 1992), which corresponds to a daily intake of live microorganisms in the range of $10^8$–$10^9$ cfu. However, as stated above, the stability of probiotic preparations is frequently impaired by factors related to the technological manufacturing process, ripening, and/or storage. In addition, not only is the maintenance of viability important in the case of probiotics, but also functional properties have to be maintained during manufacture and shelf life to deliver the probiotic effect in the GIT (Ross et al. 2002).

The sensitivity to acidic conditions, oxygen, and other environmental factors of probiotics in fermented milks varies among the different strains. In general, the species *B. animalis*, including *B. animalis* subsp. *lactis*, is more resistant to environmental stress and more stable in fermented milks than other *Bifidobacterium* species (Jayamanne and Adams 2006; Abe et al. 2009c) and therefore it has been extensively used in the manufacture of fermented milks and other dairy
products worldwide (Gueimonde et al. 2004a). The combination of LAB and probiotics could influence the viability of the other microorganisms in the product (Vinderola et al. 2002b). A positive association influencing viability has been found between some probiotics as is the case of certain Bifidobacterium and Lactobacillus acidophilus strains (Kneifel et al. 1993).

Acetic acid produced by bifidobacteria could provide an undesirable taste in fermented milks. However, this rarely constitutes a real problem in such products since bifidobacterial cultures usually show a reduced activity in milk. These microorganisms are normally used as adjuncts and yogurt starters (Lb. delbrueckii subsp. bulgaricus and S. thermophilus) are added to enhance the fermentation process. As previously mentioned, one of the main factors impairing viability and functionality of cultures is the postacidification that may occur in the finished fermented product during refrigerated storage due to the residual activity of strains, mainly from the species Lb. delbrueckii subsp. bulgaricus. Literature reports abundantly the declination of viability of probiotic bifidobacteria and some lactobacilli, particularly the species Lb. acidophilus, during refrigerated storage of fermented milks (Vinderola et al. 2000a; Gilliland et al. 2002; Oliveira et al. 2002; Vinderola et al. 2002a; Gueimonde et al. 2004a; Moreno et al. 2006; Saccaro et al. 2009). To partly counteract this deleterious effect, different combinations of Lb. acidophilus or Lb. acidophilus and Bifidobacterium spp. (AB cultures); Lb. acidophilus, Bifidobacterium spp., and Lb. casei (ABC cultures); or S. thermophilus, Bifidobacterium spp., and Lb. acidophilus (ABT cultures) could be employed (Vinderola et al. 2000b; Gueimonde and de los Reyes-Gavilán 2004; Tamime et al. 2005; Damin et al. 2008), just avoiding the use of Lb. delbrueckii subsp. bulgaricus strains. This generally results in longer fermentation times and milder final acidity. The use of non-postacidifying mutants of L. delbrueckii ssp. bulgaricus has also been reported to enhance the survival of probiotic cultures in yogurt during refrigerated storage (Ongol et al. 2007). Some S. thermophilus strains can also act as oxygen scavengers in fermented dairy products, thus improving viability of anaerobic probiotics (Ishibashi and Shimamura 1993; Lourens-Hattingh and Viljoen 2001; Talwalkar and Kailasapathy 2004).

As previously mentioned, the use of stress-resistant strains can also be useful for improving strain stability during food processing and afterward during gastrointestinal transit. Some recent studies indicated that the addition of stress-resistant probiotics neither promoted remarkable changes in the behavior of starters during manufacture nor had any detrimental effect on the sensory properties of fermented milks (Maus and Ingham 2003; Oliveira et al. 2009; Sánchez et al. 2010).

Apart from the selection and combination of appropriate strains, and the control of final pH and postacidification phenomenon, other methods have been assayed to improve viability of LAB and probiotics in adverse environments, especially in fermented dairy products. Among them are the addition of protectors and oxygen scavenger compounds, or supplements such as cysteine hydrochloride and ascorbic acids (Chandra et al. 2007), vitamin E, green tea, lemon, or pectin, among others (Jimenez et al. 2008). Prebiotics are “nondigestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon and thus improve host health” (Gibson and Roberfroid 1995). Saarela et al. (2003) indicated that the prebiotic lactulose might improve cold storage stability of Lb. salivarius. In practice most prebiotics are complex carbohydrates of vegetable origin. Apart from their potential role in the human intestinal health, efforts have been made, by using prebiotics, to improve the viability of certain probiotics in fermented products and afterward during gastric transit (Ross et al. 2005; El-Nemr and Mostafa 2007; Akalin et al. 2007). Prebiotics can also be employed as entrapping matrices for probiotics, these combined products being regarded as symbiotics (Ross et al. 2005). Cell entrapment techniques by microencapsulation of probiotics have been developed
by using polymers other than prebiotics for the improvement of bacterial viability (Woo et al. 1999; Ahn et al. 2002). This is the case of alginate and κ-carrageenan among others. After drying beads, a surface coating can be applied using gelatin or whey proteins (Reid et al. 2007). Some authors suggest that the resistance of probiotics to environmental stressing conditions can be also improved in the presence of certain single sugars (Perrin et al. 2000; Noriega et al. 2006).

Cheese has also been used as a carrier for probiotic bacteria. Its higher pH, more consistent matrix, and higher fat content than fermented milk can act as more effective protecting factors for these microorganisms during storage, as well as during gastrointestinal transit (Stanton et al. 1998; Boylston et al. 2004). However, as negative factors affecting viability, water activity, cooking of curds, and long ripening times should be mentioned depending on the manufacturing process and characteristics of each particular type of cheese (Vinderola et al. 2009). In this way, fresh cheeses seem to partly overcome these problems since they are unripened, storage occurs at refrigeration temperatures, and they have a short shelf life. It is also important to determine the time and the way of adding probiotic bacteria to cheese in order to avoid their loss during whey drainage after curd formation. This, as well as the choice of suitable probiotic strains maintaining viability as well as functionality during storage, should be considered for each particular cheese (Ross et al. 2002; Vinderola et al. 2009). The addition of probiotics having metabolic activity during curd formation can cause changes in the final pH, proteolysis degree, and other technological parameters in cheeses (Bergamini et al. 2005; Milesi et al. 2009). Examples are reported in literature on the successful incorporation of probiotics to different types of fresh cheeses such as Cottage, Argentine fresh, Crescenza, and Petit-suisse (Blanchette et al. 1995; Vinderola et al. 2000c; Burns et al. 2008; Cardarelli et al. 2008) as well as ripened cheeses such as Cheddar (Stanton et al. 1998; Philipps et al. 2006), Gouda (Gomes et al. 1995), or Argentinean semihard cheese (Bergamini et al. 2005), among others.

Different varieties of ice cream and frozen dairy products have been used as carriers for probiotics. Frozen temperatures of these products are the main advantage for the stability of probiotics. $a_w$ and high osmotic pressures due to added sugars and other sweeteners could be, in contrast, an important cause of losing viability (Basyigit et al. 2006; Cruz et al. 2009; Kim et al. 2009).

### 18.3.2 Stability in Other Food Matrices

LAB can be also found in nondairy fermented substrates. Among meat products, sausages that are processed by fermenting, and without heating, are the most abundant fermented products. Microorganisms acting as starters are basically different species of lactobacilli, molds, and yeasts that multiply well in this environment and help extend shelf life and improve safety and sensory properties of fermented products. In addition, meat is a good vehicle for probiotics, providing them with a buffered environment and acting as protectors against the action of bile during digestion (Gänzle et al. 1999; Rivera-Espinoza and Gallardo-Navarro 2010).

Fruits and vegetables contain beneficial nutrients such as minerals, vitamins, dietary fiber, and antioxidants, and have taste profiles compatible with all population age groups, which make them a suitable vehicle for LAB and probiotics. LAB are the main microorganisms responsible for the natural fermentation of vegetables, strains of species belonging to *Lactobacillus* and *Leuconostoc* being the most common bacteria in natural vegetable lactic acid fermentation (Yan et al. 2008). In spite of the low pH of many fruit juices, probiotics can be used as supplements in fruit products such as tomato, carrot, and pineapple due to the good tolerance of acidic conditions by microorganisms in these particular environments (Yoon et al. 2004; Sheehan et al. 2007b). The growth and viability of microorganisms in fruits and vegetables depends on the strain, final acidity, and lactic and acetic...
acid concentrations of the product (Yoon et al. 2004). Several technologies could help to mask off-flavors that the growth of some lactobacilli can produce occasionally in some fruit juices.

Cereal grains, including soya, are important sources of protein, carbohydrates, vitamins, and minerals for many people all over the world. In addition, they are a source of nondigestible carbohydrates that can act as prebiotic substrates and/or present other additional beneficial properties such as the hypocholeresterolemic effect attributed to β-glucan. Grains are also sources of phytochemicals such as phytoestrogens, phenolic compounds, antioxidants, phytic acid, and sterols. The nutritional quality of whole grains is, however, lower than milk, fish, or meat because of the lower protein content, deficiency in essential amino acids (lysine, threonine, and tryptophan), low starch availability, and presence of antinutrients (phytic acid, tannins, and polyphenols). Cereals represent a good substrate for the growth and stability of LAB. Fermentation in traditional and nontraditional fermented cereal products decrease the level of some polysaccharides, improve the protein quality by bacterial synthesis of some amino acids, and increase the availability of group B vitamins. Fermentation also provides optimum pH conditions for enzymatic degradation of phytate and the release of minerals from fiber (Charalampopoulos et al. 2002; Kalui et al. 2010; Rivera-Espinoza and Gallardo-Navarro 2010).

18.3.3 Stability in Dry Form

Dried probiotics exhibit a wide variability in their stability. Normally, different species belonging to the same genus can exhibit different behaviors during storage in dry form (Carvalho et al. 2004). In the case of dry products, shelf life is directly related to the survival and stability of the probiotic strains included. Generally, viability of the strains decreases during storage (Abe et al. 2009b). Probiotic stability is negatively affected by high $a_w (>0.25)$, and elevated storage temperatures, as well as by the presence of oxygen (Teixeira et al. 1995; Anal and Singh 2007). Among them, a high $a_w$ seems to be highly detrimental even when bacteria are included encapsulated (Weinbreck et al. 2010). Thus, in general, the higher the $a_w$, the lower stability observed in the product (Abe et al. 2009b).

Removal of water due to drying seriously damages the cytoplasmic membranes and associated proteins, which leads to a substantial loss of microbial viability. Stability of probiotics in dry form is thus directly related to their thermal and osmotic resistance (Rokka and Rantamaki 2010). In addition, stability is also dependent on the drying technique, and thus carrier type and concentration, as well as outlet temperature, are key parameters in spray drying (Lian et al. 2002; Ananta et al. 2005).

Drying processes have an overall survival rate of 70–85%, but have the disadvantage that strain stability and viability decrease during product storage (Rokka and Rantamaki 2010). To improve cell survival, addition of deoxidants, dessicants, sugars, or milk fat have been tested (Lian et al. 2002; Picot and Lacroix 2004; Ananta et al. 2005). In this way, the use of a cryoprotectant can increase, up to 100 times, survival after freeze-drying (Godward et al. 2000; Godward and Kailasapathy 2003).

Thus, the physicochemical parameters conditioning the drying process have to be carefully analyzed for obtaining stable and viable probiotic bacteria in dry form (Yu et al. 2006).

18.4 Methods for Determining Stability

In spite of the increased availability of fast and reliable culture-independent molecular tools for the identification and quantification of LAB and probiotic microorganisms in commercial products,
manufacturers and regulatory bodies commonly use traditional culture techniques to evaluate the stability of the strain(s) in the product. In addition, culture-dependent techniques are still crucial to isolate the strains used in order to determine any physiological or biochemical change in the strain during the storage of the product. Moreover, isolation and identification of strains is also important for quality control purposes, and should be done routinely, as a number of reports have indicated that the identity of recovered microorganisms does not always correspond to the information stated on the product label (Temmerman et al. 2003a; Gueimonde et al. 2004a; Aureli et al. 2010). Nevertheless, these culture-dependent methods show several limitations, which, together with the advance of molecular biology, have lead to the recent development of culture-independent techniques that overcome some of the limitations of the culture-based approaches (Figure 18.2).

18.4.1 Culture-Dependent Methods

Differential enumeration of probiotic and starter bacteria is a complex issue. Very often microorganisms are included in foods or supplements in combination with other LAB, hindering the enumeration of the specific strain in the product. The quantification of probiotics and LAB has been traditionally carried out by plate counting. Different selective and differential media have been used for counting bacteria in food products or supplements. The choice of the media is depending on the target microorganism. Differential media allow the growth of different microorganisms, but make possible the differentiation of the specific microorganism of interest. Selective media take advantage of specific features of the microorganism of interest, which allow, or favor, its growth over other bacteria. However, it is important to underline that selective media may limit the growth of sublethally injured cells, due to the presence of the selective agents, which may lead to an underestimation of the population size. The culture media used may also differentially affect the recovery of the different species (Apajalahti et al. 2003), leading to the overestimation of certain species and the underestimation of others. Very often media are based in a metabolic feature, that is, fermentation of a certain sugar, present in the microorganisms of interest, but not
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in others that may be present in the product (ISO 20128/IDF 192; Lankaputhra and Shah 1996; Shah 2000; Vinderola and Reinheimer 1999). Media based on other properties of the microorganisms, such as resistance to specific antimicrobial compounds, have also been designed for LAB and bifidobacteria (Sozzi et al. 1990; Lim et al. 1995; Ingham 1999; Vinderola and Reinheimer 1999; Shah 2000; Roy 2001; Leuschner et al. 2003; Van de Casteele et al. 2006). International standard methods for sample preparation and enumeration of certain species of LAB and bifidobacteria in milk and dairy products have been developed by the International Dairy Federation (ISO 8261/IDF 122; ISO 20128/IDF 192; ISO 29981/IDF220; ISO 7889/IDF 117). However, these media and culture conditions may not always be suitable, or applicable, to other food products.

An alternative is the use of nonselective culture media followed by specific colony identification and counting. This method avoids the use of selective agents that may be toxic to injured or stressed cells, but requires further steps to specifically identify the colonies of the targeted microorganism to allow enumeration. To date, several LAB species can be quantified by colony hybridization using labeled DNA as a specific probe (Cocconcelli et al. 1996; Martinez et al. 1998; Sujaya et al. 2002). Alternatively, specific immunoblotting methods can also be used (Duez et al. 2000).

Another technique that has been used for determining bacterial levels in foods is the most probable number method (MPN). MPN is a qualitative technique for the quantification of bacterial populations, based on the determination of the absence/presence of specific attributes of the bacterium (gas production, sugar fermentation, etc.). Bacterial concentrations are estimated by comparing the profile of the absence/presence of the specific attribute in different dilutions of the sample. This technique may be an alternative strategy when establishing accurate bacterial counts is not feasible by plate counting, or for estimating very low microbial population sizes (<100 CFU/g). On the contrary, it has been shown that MPN has an important bias toward overestimation of microbial levels (Garthright 1993) and it is not as accurate as the classical plate counts. Although extensively used for pathogens, the application of this method for probiotics and LAB is scarce (Giraud et al. 1998; Szajewska et al. 2004; Bibiloni et al. 2001).

Plate counting and the other culture-dependent methods are based on the assumption that the culture media and the culturing conditions used are optimal for the targeted strains in all situations and allow the growth of all cells, which may not always be the case. Therefore, cells that may be viable but are not able to form colonies under standard conditions, are not counted. Moreover, storage in unfavorable conditions, such as in a fermented product with a low pH, may over time stress the microbes and change their metabolism from active growth state toward survival state, or may even lead to cell injury, thereby affecting the plate counts.

18.4.2 Culture-Independent Methods

Several culture-independent molecular methods such as DNA microarrays, polymerase chain reaction (PCR), temperature gradient gel electrophoresis (TGGE), and denaturing gradient gel electrophoresis (DGGE) analyses can be used for fast and specific detection of bacterial species. TGGE and DGGE are useful for qualitative analyses of nucleic acids and have been used to study the microbiological composition of food products (Masco et al. 2005; Temmerman et al. 2003b). Nevertheless, these methods do not provide accurate quantitative data.

Molecular enumeration methods have also been developed during the last few years. The most widely used approaches are fluorescent in situ hybridization (FISH) and real-time quantitative PCR (qPCR).

FISH is based on labeling cells with ribosomal RNA (rRNA)-targeting fluorescent probes specific for a certain microbial group. Microbes can then be detected by using, for instance,
fluorescence microscopy or flow cytometry. A limitation of this method is that to label the cells with specific probes, the microorganisms need to be fixed, and therefore analysis of cell viability is not feasible. FISH technique has been used, among other techniques, for enumeration of bifidobacteria and LAB in foods (Sohier and Lonvaud-Funel 1998; Lahtinen et al. 2006a).

Among the wide range of molecular methods, the techniques based on PCR have been most widely used for enumeration of LAB and bifidobacteria in foods (Furet et al. 2004; Lahtinen et al. 2006b; Masco et al. 2007). PCR is based on the use of a pair of primers that are complementary to a defined DNA sequence, which allows the design of primers specific for certain microorganisms. In qPCR, the DNA being amplified is quantified in real time during the PCR reaction. It is then possible to identify and quantify specific species, or bacterial groups, in a monoculture, or in complex microbial communities by qPCR. Several primers have been designed in this way for the identification and quantification of *Bifidobacterium* (Gueimonde et al. 2004; Lahtinen et al. 2005; Youn et al. 2008) and LAB (Furet et al. 2004b; Sheu et al. 2009; Tsai et al. 2010) species. A critical step in qPCR is the isolation and purification of DNA, which very often is difficult in complex matrices such as foods.

### 18.4.3 Viability-Oriented Assays

Unfortunately, most molecular methods are not suitable for differentiation between viable and nonviable bacteria. Hence, modifications of the qPCR method have been proposed to enable fast qPCR-based viability measurements in foods. Propidium monoazide (PMA) can only pass into cells through compromised cell membranes (i.e., dead cells). Inside the cell, PMA intercalates into the DNA and upon exposure to light will covalently bind to it, inhibiting PCR amplification (Rudi et al. 2002; Garcia-Cayuela et al. 2009). These methods require a careful setup and optimization to avoid PCR product amplification from dead cells and to ensure that the treatments used do not compromise the PCR reaction of the DNA isolated from viable cells.

Another option for viability assessment is the use of RNA as a target molecule. The molecules proposed as targets include rRNA and messenger RNA (mRNA). rRNA has been assessed as an indicator of microbial viability (Molin and Givskov 1999; Lahtinen et al. 2008). However, in normal conditions, the half-life of rRNA is relatively long and, therefore, rRNA is not considered a good viability marker (Lahtinen et al. 2008). Since mRNA is a highly labile molecule with a short half-life, it has been suggested as a target molecule for viability assays. mRNA can be expected to provide a better correlation for indication of viable bacteria than other nucleic acids, although it has been shown that, under some conditions, mRNA may be detectable hours after the cells have died (Birch et al. 2001). Viability assays based on the detection of mRNA of the S-layer protein of *Lactobacillus* as a target have been reported (Fitzsimons et al. 2003; Saito et al. 2004) and more recently other housekeeping genes mRNAs have been used for viability assessment of *Bifidobacterium* (Reimann et al. 2010). Nevertheless, the short half-life of mRNA also makes the use of this molecule technically challenging. The most commonly used amplification techniques for detecting RNA are reverse transcriptase PCR (RT-PCR) and nucleic acid sequence–based amplification (NASBA).

Fluorescence-based methods have been the most widely used for viability assays in the field of probiotics and LAB. Several fluorescent dyes and fluorescent-labeled probes are currently available for quantification of viable cells. Cells are first stained with a fluorescent dye, which specifically stain live and/or dead cells depending on certain characteristics typical of either viable or dead cells. Then, the fluorescence can be measured with a suitable device such as fluorescence microscope, fluorometer, or flow cytometer. Currently used targets of fluorescence-based viability
measurements include cytoplasmic membrane integrity, intracellular enzyme activity, intracellular pH, and cell reductase activity (Lahtinen et al. 2006a, b). Viability assays based on cytoplasmic membrane integrity of probiotics and LAB have been widely used (Auty et al. 2001; Lahtinen et al. 2005; Lahtinen et al. 2006a, b; Rault et al. 2007; Kramer et al. 2009). Other targets, such as intracellular enzyme activity (Bunthof et al. 2000; Lahtinen et al. 2006a), ability to maintain intracellular pH (Lahtinen et al. 2006a), or membrane potential of bacterial cells (Ben Amor et al. 2002) have also been assessed.

18.5 Conclusions

Developing and/or manufacturing a stable product containing live microorganisms such as LAB or bifidobacteria is always a challenging issue. Several factors, starting from strain properties and growing conditions, and finishing with the storage of the elaborated product, affect the stability of the microorganisms. It is therefore necessary to have a careful case-by-case selection and assessment of the raw material composition, additives used, manufacturing processes, strains used, and storage conditions to get a product in which the microorganisms added have the desired stability.

The way stability is defined and how to measure it is a topic of current discussion. Traditionally, a microorganism was considered viable if it was able to multiply in an appropriate medium. For viable cell enumeration, culture-based methods have been largely used. However, certain microorganisms readily cultivable can also exist in another state, retaining some metabolic activities typical of viable cells, and may return to being cultivable under certain conditions. This is the case of the so-called viable-but-nonculturable cells, dormant cells, or sublethally injured cells. While in pathogenic and environmental bacteria the phenomenon of the different states of viability has received quite a lot of attention, these studies are considerably less developed in probiotic bacteria. For instance, several Bifidobacterium strains have been shown to become dormant during storage of fermented products (Lahtinen et al. 2005) or sublethally injured following a stress treatment (Ben Amor et al. 2002). Therefore, methods measuring multiplication as the sole criterion of viability, although extensively used, hinder the detection of temporally uncultivable cells. For this reason, viability assays apart from those based on multiplication in culture media have been developed. The most currently used methods for measuring viability are based on fluorescence techniques. Generally such techniques use two fluorochromes allowing discrimination between intact or viable cells, injured or damaged cells, and dead cells. The availability of these methods promises to expand our understanding of bacterial viability, which may be of special importance in this field.

Finally, understanding stability, not just as viability or survival, but also in terms of changes in strain properties, is of special interest. The extensive use of LAB and bifidobacteria as probiotics requires an in-depth knowledge of the stability of the properties that allow the strains to interact with the host and to exert their beneficial effects on human health.

References


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ISO 20128/IDF 192. Milk products—Enumeration of presumptive *Lactobacillus acidophilus*—Colony count technique at 37°C.

ISO 29981/IDF 220. Milk products—Enumeration of presumptive bifidobacteria—Colony count technique at 37°C.

ISO 7889/IDF 117. Yogurt—Enumeration of characteristic microorganisms—Colony count technique at 37°C.

ISO 8261/IDF 122. Milk and milk products—Preparation of samples and dilutions for microbiological examination.


Lactic Acid Bacteria: Microbiological and Functional Aspects


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Chapter 19

Lactic Acid Bacteria in the Gut

Maria Stolaki, Willem M. De Vos, Michiel Kleerebezem, and Erwin G. Zoetendal

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19.1 Introduction

From all bacterial groups, the lactic acid bacteria (LAB) are probably the group of bacteria that is most associated with human lifestyle. The term LAB mainly refers to the ability of these organisms to convert sugars to lactic acid. The LAB comprise nonsporing, aerotolerant, coccus or rod-shaped, gram-positive, polyphyletic bacteria. The vast majority of the LAB belong to the phylum *Firmicutes*, a very diverse group of bacteria with a low (<50 mol%) G+C content in their genomes and includes the genera *Aerococcus*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Lecunostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Carnobacterium*, *Tetragenococcus*, *Vagococcus*, and *Weissella* species (Wood and Holzapfel 1995). The genus *Bifidobacterium* is also considered by many scientists as a member of the LAB as it shares some of its typical features, such as the production of lactic acid. However, the genus *Bifidobacterium* belongs to the phylum *Actinobacteria*, a group of bacteria with a high (>50 mol %) G+C genomic content and has a different mode of sugar fermentation compared with the LAB genera in the *Firmicutes* phylum, and will therefore not be discussed in this chapter.
The LAB play a prominent role in the world’s food supply, having a leading part not only in the fermentation of dairy products, meats, and vegetables, but also in the production of wine, coffee, silage, cocoa, sourdough, and numerous indigenous food fermentations (Wood and Holzapfel 1995). In addition to their role in the food production, LAB are also associated with our mucosal surfaces, in particular with the gastrointestinal (GI) tract, and oral and vaginal cavities of humans and other mammals (Wood and Holzapfel 1995, and other chapters in this book). Therefore, because of their Generally Accepted As Safe (GRAS) and Qualified Presumption Safety (QPS) status and based on their common presence in daily human diets, LAB have received tremendous attention with respect to their potential health-promoting properties. Lactobacilli and bifidobacteria have been linked to human health since the late 1800s when Metchnikoff (1907) first proposed that LAB may have a beneficial effect on humans. Metchnikoff proposed that LAB in fermented milk could promote the development and maintenance of a healthy intestinal microbiota; in particular they could prolong human life by preventing putrefaction. In the 21st century, lactobacilli and bifidobacteria have an important position in the market of so-called functional foods, including probiotic products that contain “live microorganisms which when administered in adequate amounts confer a health benefit on the host” (FAO/WHO 2002). In this chapter we focus on the diversity and potential role of LAB in the human GI tract.

19.2 Intestinal LAB

The vertebrate GI tract, including that of humans, harbors along its length a vast collection of microbes, mostly bacterial species, that is referred to as the gut microbiota. Within the GI tract different microbial habitats are recognized starting from the oral cavity, proceeding through the esophagus to the stomach and the small intestine (duodenum, jejunum, and ileum) and ending in the large intestine (cecum and colon). Microbial numbers are relatively low in the stomach and the upper small bowel due to the pH of the stomach contents, the toxicity of bile salts, and the relatively high flow of the digesta (Tannock 1995). The population density increases along the GI tract with approximately $10^9$ microbes per milliliter of luminal content in the duodenum, $10^8$ microbes per gram ileum content, and up to $10^{12}$ microbes per gram of colonic content (O’Hara and Shanahan 2006; Booijink et al. 2007). The diversity of the gut microbiota is impressively huge and has only recently been recognized at its full depth with the development of culture-independent 16S rRNA gene–based approaches. These methods have indicated that the vast majority of the intestinal microbiota belongs to three phyla, the Firmicutes; including the large class of Clostridia and the Bacilli that encompass the LAB; the Bacteroidetes; and the Actinobacteriacaea, which includes the genus Bifidobacterium (Zoetendal et al. 2008).

19.2.1 Lactobacilli in the GI Tract

Of all the GI tract–associated LAB, bacteria belonging to the genus Lactobacillus have been recognized as common inhabitants of the GI tract of vertebrate animals and they have received considerable attention in the last decades due to their postulated health-promoting properties. Lactobacilli are gram-positive non-spore-forming rods, catalase negative when growing without a heme source (e.g., blood), usually nonmotile, and occasionally nitrate reducing (Hammes and Hertel 2006). Taxonomically, the genus Lactobacillus belongs to the phylum Firmicutes, class Bacilli, order Lactobacillales, family Lactobacillaceae. They are nutritionally fastidious, requiring rich media to grow including the availability of carbohydrates, amino acids, peptides, fatty acid
Lactic Acid Bacteria in the Gut

Lactic acid bacteria (LAB) are found in a variety of carbohydrate-rich habitats such as mucosal surfaces of humans and animals, materials of plant origin, manure, sewage, and fermented or spoil food products (Stiles and Holzapfel 1997). The *Lactobacillus* genus holds more than 125 species and encompasses a wide variety of organisms, including dairy exclusive organisms (e.g., *Lactobacillus delbrueckii* ssp. *bulgaricus* and *L. helveticus*), organisms commonly found in vertebrate GI tracts (e.g., *L. acidophilus* and *L. gasseri*), and organisms with considerable adaptability to diverse habitats (e.g., *L. plantarum* and *L. casei*) (Kandler and Weiss 1986). They derive almost all of their energy from the fermentation of sugars, and based on their fermentative characteristics they are divided into homofermentative and heterofermentative lactobacilli. The homofermentative lactobacilli convert available sugars only to lactic acid while the heterofermentative lactobacilli produce, in addition to lactic acid, CO₂ and ethanol or acetate.

Over the last decades a wide variety of studies have reported the enumeration and detection of lactobacilli in human intestinal samples using culturing techniques as well as 16S ribosomal RNA (rRNA)-based approaches. Most studies using culturing approaches have reported numbers of approximately 10⁶ colony-forming units (CFU) per gram (Finegold et al. 1974; Tannock et al. 2000; Walter et al. 2001; Dal Bello et al. 2003; Dal Bello and Hertel 2006), which accounts for approximately 0.01% of the total number of fecal microbes. These numbers are consistent with outcomes from culture-independent 16S rRNA (gene)-based approaches, although there is a large subject-to-subject variation, with averages of 10⁶–10⁸ cells per gram of feces (wet weight) (Harmsen et al. 2002; Rinttila et al. 2004) to subjects with numbers of lactobacilli below the detection limit (Tannock et al. 2000; Walter 2008). On the other hand, the fraction of 16S rRNA genes of *Lactobacillus* origin has been reported up to 13% in clone libraries from other locations in the GI tract, suggesting that the lactobacilli concentration is also dependent on anatomical location in the GI tract (Hayashi et al. 2005; Frank et al. 2007).

For the upper intestinal tract, there are contradicting observations with respect to the dominance of lactobacilli. However, it is noteworthy to mention that *Lactobacillus* enumerations originating from the small intestine are not widely available due to the poor accessibility of this part of the GI tract and the inability to obtain luminal samples in a noninvasive way. Cultivation approaches have indicated that lactobacilli in fact are among the most common bacteria in the stomach, duodenum, and jejunum of humans, which suggest that rich, easily available carbohydrate-containing substrates derived from the host diet are used as primary nutrient source by these lactobacilli (Bongaerts and Severijnen 2001; Reuter 2001). This is also reflected by the significant number of genes encoding carbohydrate-utilizing enzymes and putative sugar transporters, which relate to the niche they occupy within the GI tract (Claesson et al. 2007). In addition, a recent study focusing on populations in ileostomy effluent (small intestinal content collected in a stoma bag from people of whom the colon has been surgically removed) from small-bowel posttransplant patients has shown a dominance of lactobacilli and enterobacteria (Hartman et al. 2009). Moreover, another recent study focusing on ileostomy effluent demonstrated the consistent dominance of streptococci while lactobacilli numbers contributed in different percentages, ranging from less than 1% to as high as 47% depending on the subject and time of sampling (Booijink et al. 2010). In contrast, cloning and sequencing of 16S rRNA genes have indicated that lactobacilli only form a minority of the microbial community in the ileum (Wang et al. 2003; Wang et al. 2005; Ahmed et al. 2007). This enormous fluctuation indicates that subject- and environment-related factors, such as food consumption, may have a crucial impact on small intestinal populations, including the lactobacilli. The very low abundance of lactobacilli in the large intestine versus their occasionally high abundance in...
the small intestine suggests that the latter region is a nutritionally more dynamic environment that offers the opportunities to lactobacilli to adapt, interact physically with exposed surfaces of the host, and survive. Part of the contradicting observations, especially for the small intestine, may be explained by the different technologies and their inherent biases that are used to study the community structure and population dynamics of the small intestinal microbiota. However, another reason could include the difficulty to discriminate autochthonous and allochthonous populations of lactobacilli as will be discussed below.

19.3 Lactobacilli in the Gut: True Residents or Food-Derived Transients?

The indigenous lactobacilli of the human GI tract consist of autochthonous (true residents) but also of allochthonous (transient) species. The autochthonous lactobacilli are bacteria that are able to occupy a niche in the GI tract, to colonize a mucosal surface of the GI tract due to special adhesion factors including compatibility with the immunological system of the host (Reuter 2001). The autochthonous lactobacilli have to be distinguished from allochthonous species, which may also be encountered in the intestine, but may only reside in this niche in a transient manner. Allochthonous lactobacilli are bacteria mainly originating from food products and the oral cavity microbiota. The presence of these strains in the intestinal tract lasts for a limited time, probably only a few days (Tannock 1999). However, due to the wide variety of LAB in different environments, including foods, it is not easy to discriminate these groups.

Thus far, there are 17 Lactobacillus species that have been associated with the human GI tract, some of which were only recently detected by molecular techniques using PCR primers specific for LAB (Walter 2008). Particular Lactobacillus species such as L. acidophilus, L. casei, L. paracasei, L. rhamnosus, L. delbrueckii, L. brevis, L. johnsonii, L. plantarum, and L. fermentum have not been reported to form stable populations and are likely to be allochthonous. Most of these species are regularly present in fermented foods, and they are common inhabitants of the oral cavity (Walter 2008) (Table 19.1). More specifically, allochthonous lactobacilli are introduced regularly into the GI tract because they are ubiquitous in nature, especially in association with food products (Dal Bello and Hertel 2006). Thus, depending on individual consumption habits, these lactobacilli are likely to be transferred day by day through the stomach and small intestine into the large intestine, and can be detected in human feces (Walter et al. 2001; Heilig et al. 2002). Moreover, lactobacilli have also been detected in human saliva in variable numbers that go up to population levels exceeding 10^6 CFU/ml (Ahola et al. 2002). Dal Bello and Hertel (2006) have shown, by using both culture-dependent and -independent approaches, that the Lactobacillus species L. gasseri, L. paracasei, L. rhamnosus, and L. vaginalis are allochthonous to the human intestine and originate from the oral cavity. The potential continuous inoculation of lactobacilli from food and oral cavity may also explain the contradictory observations of small intestinal lactobacilli, since these factors have a more prominent influence on the small intestine microbiota compared with the colon. On the other hand, L. gasseri and its close relative L. johnsonii are often considered as the true autochthonous species of the human intestinal microbiota; L. gasseri is one of the Lactobacillus species native to human GI tract of neonates (Wall et al. 2007) and has been reported as a dominant Lactobacillus species in the colonic mucosa of a variety of individuals (Zoetendal et al. 2002). All the factors indicated above make it very difficult, often impossible, to determine whether a particular Lactobacillus species can be considered as an autochthonous inhabitant of the human GI tract.
Table 19.1  Lactobacillus Species Detected in Human Feces, Saliva, and Food

<table>
<thead>
<tr>
<th>Species</th>
<th>Feces</th>
<th>Oral Cavity</th>
<th>Food Products</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>L. acidophilus</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Walter (2008)</td>
</tr>
<tr>
<td>L. crispatus</td>
<td>Yes (in some subjects)</td>
<td>Yes</td>
<td></td>
<td>Tannock (1999); Reuter (2001)</td>
</tr>
<tr>
<td>L. gasseri</td>
<td>Yes (in some subjects)</td>
<td>Yes</td>
<td></td>
<td>Tannock (1999); Reuter (2001)</td>
</tr>
<tr>
<td>L. johnsonii</td>
<td>Yes</td>
<td></td>
<td>Yes</td>
<td>Hammes and Hertel (2003)</td>
</tr>
<tr>
<td>L. salivarius</td>
<td>Yes (in some subjects)</td>
<td>Yes</td>
<td></td>
<td>Tannock (1999); Reuter (2001)</td>
</tr>
<tr>
<td>L. ruminis</td>
<td>Yes (in some subjects)</td>
<td></td>
<td></td>
<td>Walter (2008)</td>
</tr>
<tr>
<td>L. casei</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Hammes and Hertel (2003)</td>
</tr>
<tr>
<td>L. paracasei</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Hammes and Hertel (2003)</td>
</tr>
<tr>
<td>L. rhamnosus</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Tannock (1999); Reuter (2001)</td>
</tr>
<tr>
<td>L. plantarum</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Tannock (1999); Reuter (2001)</td>
</tr>
<tr>
<td>L. reuteri</td>
<td>Yes (in some subjects)</td>
<td>Only in sourdough/fermented cereals</td>
<td>Tannock (1999); Reuter (2001); Hammes and Hertel (2003)</td>
<td></td>
</tr>
<tr>
<td>L. fermentum</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Walter (2008)</td>
</tr>
<tr>
<td>L. brevis</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Reuter (2001); Hammes and Hertel (2003)</td>
</tr>
<tr>
<td>L. delbrueckii</td>
<td>Yes</td>
<td></td>
<td>Yes</td>
<td>Reuter (2001); Hammes and Hertel (2003)</td>
</tr>
<tr>
<td>L. sakei</td>
<td>Yes</td>
<td></td>
<td>Yes</td>
<td>Reuter (2001); Hammes and Hertel (2003)</td>
</tr>
<tr>
<td>L. vaginalis</td>
<td>Yes</td>
<td></td>
<td></td>
<td>Walter (2008)</td>
</tr>
<tr>
<td>L. curvatus</td>
<td>Yes</td>
<td></td>
<td></td>
<td>Walter (2008)</td>
</tr>
<tr>
<td>S. thermophilus</td>
<td>Yes</td>
<td></td>
<td></td>
<td>Iyer et al. (2010)</td>
</tr>
</tbody>
</table>

(continued)
19.4 Lactobacilli in the Gut: Successful Adaptation

To gain insight into the genetic potential of *Lactobacillus* species and how this can be linked to their lifestyle in different environments, genome sequencing of isolates and comparative genomics analysis offer great potential to decipher these relations. Comparative genomic analysis has revealed that lactobacilli have a diverse carbohydrate metabolic potential that is regarded as one of the driving forces behind their ability to survive and function efficiently in various niches, including the GI tract. The genomes of lactobacilli encode specialized saccharolytic machinery that reflects the nutrient availability in respective environments (Kleerebezem et al. 2003; Pridmore et al. 2004; Klaenhammer et al. 2005; Kant et al. 2010). To date, 33 *Lactobacillus* genomes have been completely sequenced while at least 100 *Lactobacillus* genome sequencing projects are in progress (http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi). Most lactobacilli have small genome sizes ranging between 1.8 and 3.3 Mb (Klaenhammer et al. 2005). Essential information has been retrieved from these genome annotations with respect to metabolic pathways, physiology, biosynthetic capabilities, and adaptability to varying conditions and environments as has been extensively reviewed previously (Klaenhammer et al. 2005; Kleerebezem and Vaughan 2009).

Lactobacilli and other food microorganisms encounter various environmental conditions upon ingestion by the host and during transit in the GI tract. Starting from the stomach, bacteria need to survive the harsh conditions of an acidic environment in combination with the presence of

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**Table 19.1 Lactobacillus Species Detected in Human Feces, Saliva, and Food (Continued)**

<table>
<thead>
<tr>
<th>Species</th>
<th>Feces</th>
<th>Oral Cavity</th>
<th>Food Products</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. mitis</em></td>
<td>Yes</td>
<td>Yes</td>
<td></td>
<td>Nobbs et al. (2009); Takahashi and Nyvad (2011); van den Bogert et al. (2011)</td>
</tr>
<tr>
<td><em>S. mutans</em></td>
<td></td>
<td>Yes</td>
<td></td>
<td>Nobbs et al. (2009); Takahashi and Nyvad (2011)</td>
</tr>
<tr>
<td><em>S. sanguinis</em></td>
<td>Yes</td>
<td></td>
<td></td>
<td>Nobbs et al. (2009); Takahashi and Nyvad (2011)</td>
</tr>
<tr>
<td><em>S. oralis</em></td>
<td></td>
<td>Yes</td>
<td></td>
<td>Nobbs et al. (2009); Takahashi and Nyvad (2011)</td>
</tr>
<tr>
<td><em>S. gordonii</em></td>
<td>Yes</td>
<td>Yes</td>
<td></td>
<td>Nobbs et al. (2009)</td>
</tr>
<tr>
<td><em>S. sobrinus</em></td>
<td>Yes</td>
<td></td>
<td></td>
<td>Nobbs et al. (2009); Takahashi and Nyvad (2011)</td>
</tr>
<tr>
<td><em>S. bovis</em></td>
<td></td>
<td>Yes</td>
<td></td>
<td>van den Bogert et al. (2011)</td>
</tr>
<tr>
<td><em>S. intermedius</em></td>
<td>Yes</td>
<td></td>
<td></td>
<td>van den Bogert et al. (2011)</td>
</tr>
</tbody>
</table>
gastric juices. Further down, bile acids, synthesized by the liver from cholesterol, are secreted into the small intestine to facilitate a more effective digestion and absorption of food compounds, such as fat. Given that the liver excretes approximately 1 liter of bile per day into the small intestine, exposure to bile represents another challenge for bacteria of the GI tract (Begley et al. 2006). Bile acids are synthesized from cholesterol and are conjugated to either glycine or taurine. Despite the lack of concrete knowledge on the toxicity of bile acids for bacterial cells, bile acids are surface-active, amphipathic molecules with potent antimicrobial activity and act as detergents, disrupting biological membranes. Moreover, bile salts seem to induce an intracellular acidification so that many bacterial resistance mechanisms are shared between bile and acid stress responses (Begley et al. 2006). Many intestinal lactobacilli possess the ability to interact with bile salts and modify them by hydrolyzing the amide linkage between their amino acid moieties and cholesterol backbones via bile salt hydrolases (BSH) (Begley et al. 2006; Lambert et al. 2008). BSHs are the enzymes releasing taurine or glycine from bile leading to the protection of cells from the conjugated form of bile and they are almost exclusively associated with GI tract–colonizing bacteria (Begley et al. 2006). They are assumed to assign a selective advantage in the GI tract environment (Tanaka et al. 1999), which is supported by observations of Dussurget and co-authors (2002) that loss of BSH activity by the pathogen *Listeria monocytogenes* reduces its survival in the guinea pig GI tract by more than four logs, while increasing BSH copy number enhances survival by 10-fold. The distribution of genes encoding BSH among lactobacilli suggests a clear association of this function with the intestinal habitat (Lambert et al. 2008; Kleerebezem et al. 2010). *L. plantarum* WCFS1 and *L. johnsonii* NCC 533 encode the largest numbers of BSHs found in any of the bacterial genome to date (three and four, respectively) (Kleerebezem et al. 2003; Pridmore et al. 2004). However, recent functional analysis of the four genes annotated as *bsh* gene in the genome of *L. plantarum* revealed that only one of these genes actually encodes a true BSH, while the other genes appear to resemble the structurally related penicillin acylase enzyme family (Lambert et al. 2007; Lambert et al. 2008). *L. gasseri* contains similarity to a locus in *L. acidophilus* KS-13 and *L. johnsonii* 100-100 encoding a BSH (Elkins et al. 2001; Azcarate-Peril et al. 2008), which is syntenous with the sequenced genome of *L. johnsonii* NCC 533. In addition, another BSH (LGAS_0965) shows a high degree of homology with the BSH in *L. johnsonii* NCC 533 and *L. acidophilus*, with the homologue of this gene in the latter *Lactobacillus* to be shown to specifically hydrolyze bile salts conjugated to the amino acid taurine (McAuliffe et al. 2005; Jones et al. 2008). Bile-specific transporters have also been found in many lactobacilli. A study in *L. reuteri* identified an operon containing a multidrug resistance transporter (MDR) and a gene of unknown function differentially expressed in the presence of bile. When these genes were deleted, the strain demonstrated a reduced ability to recover in the presence of bile, suggesting that transport of bile salts plays an important role in bile tolerance (Whitehead et al. 2008). One of the genes of the identified two-component regulatory system operon found in *L. acidophilus* NCFM is a MDR playing important role in bile tolerance (Pfeiler et al. 2007). Bron and colleagues in their study of screening for bile-responsive genes in *L. plantarum*, identified three possible exporter proteins, including a putative MDR transporter gene (Bron et al. 2004a). Overall, lactobacilli have maintained and acquired a variety of genes that allows them to survive bile exposure, and hence genes encoding BSH can be considered as one of the few genes associated with life in the human GI tract (Jones et al. 2008) although the annotation of the BSH genes may deserve some more attention to appropriately assign this function to individual species and strains of the lactobacilli (Lambert et al. 2008).

Whole genome analysis is now providing insights into the genetic basis for the adaptation of lactobacilli in widely different environments. There are a number of proposed mechanisms of niche adaptation, namely gene loss or decay, lateral gene transfer, or gene upregulation or mutation.
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(O’Sullivan et al. 2009). Many members of the *Lactobacillus* species that are strongly adapted to food fermentations appear to have a higher number of inactive genes (pseudogenes) due to frame-shift, nonsense mutation and deletion or truncation in comparison with the GI tract–associated strains (O’Sullivan et al. 2009), and it is noteworthy that the majority of them appear to be essential genes for survival in the gut, including their involvement in bile salt hydrolysis as well as carbohydrate and amino acid metabolism and transport (O’Sullivan et al. 2009). Furthermore, there is also evidence of regions of laterally transferred genes present in lactobacilli, namely recently acquired chromosomal regions or genomic islands described in *L. plantarum* WCFS1, which are thought to increase the ability of the bacterium to adapt to multiple environmental niches (Kleerebezem et al. 2003; Molenaar et al. 2005; Boekhorst et al. 2006). Kankainen and colleagues (2009) have recently demonstrated that in the genomes of a widely used probiotic bacterium (*L. rhamnosus* GG) and an adjunct starter culture exhibiting reduced binding to mucus (*L. rhamnosus* LC705), which showed high sequence identity and synteny, three canonical pilus subunits and a dedicated sortase typically required for assembling pili were present. Pili are proteinaceous cell surface appendices characterized previously in gram-positive pathogens where they are involved in host-cell attachment (Mandlik et al. 2008; Proft and Baker 2009). The findings of Kankainen and colleagues (2009) showed the presence of pili in *L. rhamnosus* GG and the mucus binding capacity mediated by the tip pilus protein of the bacterium, suggesting how *L. rhamnosus* GG may persist in the host and possibly compete with pathogens for niches in the human GI tract. The above, in combination with the acquisition of multiple transporters in order to scavenge important growth factors from their nutrient-rich environments, indicates some of the mechanisms lactobacilli have developed for their niche adaptation.

Up to date, several postgenomic studies have been performed addressing the molecular adaptation of lactobacilli to the intestinal habitat. Due to the physicochemical and microbial complexity of the intestinal environment, *in vivo* studies could give more representative outcomes as compared with *in vitro* screening conditions. To that end, *in vivo* animal studies focusing on the genetic responses of individual microbes have been conducted. *In vivo* expression technology revealed 72 GI tract induced (*ivi*) functions in *L. plantarum*, of which homologues had previously been associated with survival and adaptation in intestinal pathogens (Bron et al. 2004b). A similar approach was also employed in *L. reuteri* (Walter et al. 2003), revealing three genes that are specifically induced in the mouse intestinal tract. Furthermore, mutation analysis showed the critical contribution of some *ivi* genes such as genes encoding proteins with a predicted role in cell envelope functionality, stress response, and regulation to gut persistence (Bron et al. 2007). Moreover, transcript levels of *ivi* genes indicated variation as a function of location along the length of the mouse intestine (Marco et al. 2007).

The expression of representative genes in *L. plantarum* WCFS1 as a follow up of ingestion and passage through the human upper GI tract and small intestine was assessed by quantitative RT-PCR on RNA templates isolated from ileostomy effluent of patients who consumed these bacteria in a milk-based product (de Vries 2006). The study showed that *L. plantarum* was metabolically active as indicated by induction of genes for protein synthesis, amino acid biosynthesis, and sugar uptake and metabolism for energy to eventually lactate and ethanol production. It is worth mentioning that although no differential expression was observed for some cell surface protein genes nor for the BSH gene, a potential adherence protein was upregulated (de Vries 2006).

To further describe the bacterial behavior in the intestine, several transcriptomic approaches have been performed. When *L. johnsonii* was analyzed with transcriptomic tools by Denou and colleagues (2007), the expression of distinct gene sets was revealed when the microbe resides in different locations in the mouse intestine. Similarly, the gene transcripts of *L. plantarum* revealed significant *in situ* adaptations of its carbohydrate metabolism and cell surface properties when
compared with in vitro growth conditions (Marco et al. 2009; Marco et al. 2010). In the same study (Marco et al. 2009), the transcriptome analysis showed that the gene expression was strongly affected by the mouse diet. In general a lower growth rate and an altered carbohydrate metabolism of L. plantarum were observed in mice fed a Western-style diet compared with mice fed a typical rodent chow diet. The responses of L. plantarum WSFS1 at the murine cecum were directed toward expression of specific functional pathways in ways that were either dependent or independent of host diet, indicating its flexibility to adjust to environmental conditions of hosts and/or nutritional origin (Marco et al. 2009). All the above indicate the importance of adaptation of the nutrient utilization machinery lactobacilli carry to adapt and survive in the different substrate niches of the GI tract. Obviously, it is also of crucial importance to study all the above-mentioned parameters in vivo in humans to elucidate the in situ activity of the lactobacilli, which will ultimately assist in unraveling their molecular interactions with the host. The first attempts have been already conducted, and to that end, as shown in a recent study of Marco and colleagues using comparative transcriptome analysis, the probiotic L. plantarum 299v specifically adapts its metabolic capacity in the human intestine for carbohydrate acquisition and expression of exopolysaccharide and proteinaceous cell surface compounds (Marco et al. 2010). Furthermore, in the same study, a core of L. plantarum transcriptome expressed in the mammalian intestine was also determined through comparisons of L. plantarum 299v activities in humans to those found for L. plantarum WCFS1 in germ-free mice (Marco et al. 2010). Induction of genes encoding the transporters and enzymes for utilization of carbohydrates was observed in both humans and mice, confirming the relevance of these processes in microbial adaptation to the intestinal ecosystem. New Lactobacillus genomes are becoming available with an increasing rate (van de Guchte et al. 2006; Callanan et al. 2008; Cai et al. 2009), resulting in emergence of species’ pan-genomes. This explosion of genetic information in combination with a variety of comparative methods aids scientists to explore the features of individual sequenced Lactobacillus species and strains. The up-to-date available genome sequences of lactobacilli suggest their dynamic and flexible character in regard to survival and adaptation in their niches. However, as indicated above, it remains a challenge to identify “gut-specific genes” because of the difficulty to discriminate between true autochthonous and allochthonous lactobacilli. Nevertheless, the fast developments in sequencing technologies in combination with the increase in metagenomics data may provide new possibilities to link specific genes to environment as these allow the study of ecosystem-specific pan-genomes rather than genome sequences of specific isolates.

19.5 Streptococci: Forgotten LAB in GI Tract

A majority of studies focusing on LAB in the intestine have focused on lactobacilli. However, numbers of LAB are very low in the colon and are strongly fluctuating in the small intestine. In contrast several studies have demonstrated that another genus of the LAB, namely Streptococcus, is dominantly detected in both small intestinal effluent (Booijink et al. 2010) as well as in mucosal biopsies (Wang et al. 2005), especially of the proximal small intestine. Moreover, it was recently demonstrated that Streptococcus sp. are basically always detected in significant numbers despite the high population dynamics of the small intestinal ecosystem, which has not been demonstrated for the Lactobacillus genus (Booijink et al. 2010). The same study also demonstrated the existence and abundance of streptococci, especially S. bovis–related species, in the small intestine of human subjects. Furthermore, the single reported study that aimed to describe the microbiota residing in the luminal part of the human ileum and used mucosal tissue samples collected from sudden
death victims, revealed a relative abundance of the genera *Bacillus*, *Streptococcus*, and *Lactobacillus* (Hayashi et al. 2005). These studies point out that streptococci are more “typical” members of the small intestine than lactobacilli, and hence a detailed look at this group of LAB in relation to the small intestinal ecosystem deserves more attention.

Streptococci comprise a variety of diverse species, including harmful pathogens such as *S. pyogenes*, *S. agalactiae*, and *S. pneumoniae* but also species that have the GRAS status, such as *S. thermophilus*. Streptococci are identified as anaerobic or aerotolerant, catalase-negative, and gram-positive bacteria, growing as linear chains or ovoid cells (Moschetti et al. 1998). Certain members of the pathogenic streptococci such as *S. agalactiae* usually behave as commensal organisms that colonize the GI or genital tract of 25–40% of healthy women (Tettelin et al. 2001; Schuchat and Wenger 1994). *S. agalactiae* may cause life-threatening invasive infection in susceptible hosts such as newborn infants and pregnant women, which suggests that an important exhibited feature of this organism is its ability to survive and grow in diverse host environments that differ with respect to the physiological conditions and immune defenses (Seepeersaud et al. 2006).

Up to date, the number of complete streptococcal genomes available is particularly high due to the medical importance of several species such as *S. pneumoniae*, *S. pyogenes*, *S. agalactiae*, and *S. mutans*, but genomes from typical gut isolates are lacking. More than 30 genomes of *Streptococcus* are available in the public databases that belong to “only” nine different species, including *S. pneumoniae*, *S. agalactiae*, *S. pyogenes*, *S. mutans*, *S. suis*, *S. sanguinis*, *S. gordonii*, *S. equi*, and *S. thermophilus* (Maruyama et al. 2009). The study of the genomes of streptococcal species has indicated that new genes were acquired during evolution by lateral gene transfer, which provided the bacteria with a readily available novel gene pool supplying them with additional helpful physiological properties for exploiting new niches (Marri et al. 2006).

The determination of the complete genome sequence of several streptococci members has revealed their possession of various sugar transport mechanisms and a fast sugar metabolism, which make them well-equipped competitors for substrates during the fast transit through the small intestine (Milinovich et al. 2008; Booijink 2009). Global transcription profiles of *S. mutans* revealed that phosphotransferase (PTS) sugar transport systems were responsible for transport of monosaccharides (glucose, fructose, galactose, and mannose), disaccharides (sucrose, lactose, maltose, and trehalose), β-glucosides (cellobiose), and sugar alcohol (mannitol) (Ajdic and Pham 2007). Interestingly, five PTS transporters were consistently highly transcribed and constantly expressed, suggesting that their substrates, glucose, fructose, maltose, and sucrose, might be preferable sugars for this organism (Ajdic and Pham 2007). All inducible PTs showed very low transcription in the absence of the specific sugar substrate, indicating their high specificity for these particular sugars. The capacity of *S. mutans* to rapidly transport and metabolize a wide range of sugars, whenever they become available, may highlight the capacity of this species to survive in nutrient-rich niches, which includes the small intestine.

In gram-positive pathogens such as streptococci, the existence of pili formed by covalent polymerization of adhesive pilin subunits suggested the ability of the streptococci to adhere and attach to host cells, an essential step in the pathogenic process (Telford et al. 2006), similar to the recently discovered mucus-binding pili found on the surface of the nonpathogenic *L. rhamnosus* GG, which reveals a mechanism for the interaction of the bacteria with host tissues (Kankainen et al. 2009). *S. pneumoniae*, a close relative of *S. agalactiae*, is notable for its large array of PTS transporters, which may be important for utilization of host-derived carbohydrates and for recycling of capsule constituents (Tettelin et al. 2001). Another *Streptococcus* species, *S. bovis*, utilizes starch and sugars to acquire energy and the rate of sugar transport may affect its growth rate. In *S. bovis* the PTS sugar transport system is an important route for the transport of sugars and is principally...
used at low sugar concentrations (Asanuma et al. 2004; Vadeboncoeur and Pelletier 1997). To survive in vivo and colonize as a biofilm, the oral habitant S. gordonii has developed highly controlled sugar metabolism systems. When multiple sugars exist in the environment, bacteria first consume primary sugars, such as glucose, unless these are exhausted or are unavailable. In the latter case, bacteria induce genes for metabolizing secondary sugars, including β-glucosides. In the case of S. gordonii, it possesses three PTS sugar transporters and one binding protein–dependent sugar uptake system for metabolizing multiple sugars, including β-glucosides, which not only contribute to in vitro adhesion and biofilm formation but also provide energy and carbon sources for survival in the host (Kilic et al. 2004).

Similar to lactobacilli, streptococci have demonstrated high level of flexibility in adaptation to various environments. A good example is the bacterium S. thermophilus, a major dairy starter used for the manufacture of yogurt and cheese. S. thermophilus is closely related to other streptococcal species including several human pathogens (e.g., S. pneumoniae and S. pyogenes). It is therefore remarkable that S. thermophilus has a long history of safe use in food production and an undisputed GRAS status in the United States and a QPS status in the European Union (Iyer et al. 2010). It belongs to the viridians streptococci, which include 26 species, all of them encountered as commensals in the oral and GI cavities and genital tracts of mammals (Hols et al. 2005). The access to genome sequences, comparative genomics, and multilocus sequence analyses suggests that S. thermophilus recently emerged and it is still undergoing a process of regressive evolution toward a specialized bacterium for growth in milk (Boilotin et al. 2004; Hols et al. 2005; Delorme 2008). This dairy bacterium has maintained a well-developed nitrogen metabolism while its sugar catabolism has strongly degenerated. Moreover, carbohydrate metabolism of S. thermophilus is highly adapted to growth in lactose-containing environments, including a highly effective lactose–galactose antiporter LacS (Poolman et al. 1990) and a catabolite repression system that seems to be predominantly controlled by lactose as a preferred carbon source (van den Bogaard et al. 2000).

S. thermophilus has lost many virulence-related functions common among pathogenic streptococci that play important role in cell adhesion, host invasion, or escape from the immune system. Despite the gene decay in the S. thermophilus genome, small genomic islands seem to be acquired by lateral gene transfer and these regions encode a number of important industrial traits such as bacteriocin production, polysaccharide biosynthesis, or oxygen tolerance (Hols et al. 2005). Considering these remarkable differences between closely related streptococci and their respective niches, it is evident that more studies on intestinal streptococcal isolates are urgently needed to gain insight into their live and function in the intestine.

The capacity to rapidly ferment sugars in combination with the flexibility in sugar transport systems observed in streptococci makes these organisms suitable candidate residents of the small intestine, where readily available carbon sources are abundant and the transit rate is relatively high. A recent study using phylogenetic mapping of the small intestinal metagenome of three different ileostomy effluent samples from a single individual indicated that Streptococcus sp. were among the most abundant microbial groups in the small intestine and in combination with metatranscriptome analysis showed the high level of in situ expression of PTS and carbohydrate metabolic genes mainly belonging to Streptococcus sp. (Zoetendal et al. 2011). Streptococci ferment carbohydrates to produce lactate, which can be fermented in secondary fermentations by other species such as Veillonella to acetate and propionate (Foubert and Douglas 1948). The abundance of Veillonella species in the human ileum has been recently reported by Booijink and colleagues (2010) in combination with the high abundance of streptococci, which also suggests the interplay between these species, profiting in carbon sources from each in a competitive environment. In addition, Zoetendal and colleagues (2011) indicated recently with their study based on
metagenome, transcriptome, metabolite, and community profiling data that the small intestine communities are shaped by the capacity for fast import and conversion of simple carbohydrates and rapid adaptation to the niche nutrient availability, profiles that match well with streptococci. To date, a limited number of studies have focused on the interactions between dominant species in the small intestine, and the key roles these species fulfill in this habitat. Further research with the help of genome sequencing and other advanced technologies is required to shed light on the LAB present in the human small intestinal niche and their functions and interactions with the host.

19.6 Conclusions and Perspectives

LAB are typical members of the human intestine, and especially lactobacilli have received considerable attention with respect to our food production and their putative health-promoting properties. LAB are found to be more dominantly present in the small intestine than in the colon. Nevertheless, research has been mainly focused on Lactobacillus species, while the Streptococcus species, based on recent studies, are shown to be the dominant species in the small intestine and remain still largely unexplored. More studies are required to investigate the role of streptococci in the small intestine by focusing on sequencing of more streptococcal isolates from this habitat and discovering their function in specific niches within the upper GI tract.

However, the inaccessibility of the human small intestine due to the invasiveness of the sampling methods is an important barrier in the increase of knowledge of this specific niche. Obviously, in vivo studies would enhance our knowledge of the small intestinal microbiota and their dynamic interactions with the mucosa and dietary components. Based on the limited accessibility of the small intestine, this knowledge may, in part, be derived from readily accessible in vitro models that aim to simulate the physicochemical dynamics of the small intestine. This would allow the study of the small intestine microbiota members in a simple but representative way. Several model systems have been developed to study microbial diversity and functionality in the small intestine in a simplified manner (Molly et al. 1994; Minekus et al. 1995; Minekus et al. 1999). Currently, a prototype of an in vitro model system simulating the human ileum using human microbiota inocula has been developed, and initial experiments with this model have been performed. The use of such a model system may enable studies on the diversity but also on functional aspects of a complex microbial community resembling that of the human ileum in a controlled manner. The stable, diverse, and complex microbiota within the in vitro model system provides the opportunity to evaluate the impact of perturbations in the system on both microbiota composition and functionality levels. Perturbations may include manipulation of different internal parameters such as mucin presence, different oxygen levels, and transit rates. These approaches in combination with metagenomic and metatranscriptomic approaches should decipher the function of the microbiota in the small intestine, in particular that of streptococci, while more knowledge will be obtained regarding their interplay with other microbial members of the small intestinal community and their interaction with the host. The fast developments in next-generation sequencing technologies have already enabled the study of LAB pan-genomes and even the human intestinal microbiome (Qin et al. 2010) at a functional level. These developments may eventually reveal the role of the intestinal LAB in the ecosystem of the human small and large intestine. While the application of post-metagenomic approaches, such as metatranscriptomics and metaproteomics, will unravel their functional properties in this ecosystem, providing insights into the phylogeny and activity of the intestinal bacteria, including LAB in relation to the health status of the host, as well as the impact of diet, lifestyle, and other exogenous factors.
References


Chapter 20

Lactic Acid Bacteria in Oral Health

Jukka H. Meurman and Iva Stamatova

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20.1 Introduction

The oral cavity, which is the main entry to the gastrointestinal tract, can also be regarded as a doorway for good health and wellness in all the body systems. The human mouth is a unique and complex system of tissues and organs involved in nutritional, respiratory, and communicative functions. Oral health is an essential contributor to overall health (Thorstensson and Johansson 2009). Strong evidence exists for the relationship between the effects of chronic oral inflammation and general health. A well-balanced and healthy state of the mouth is maintained by the intricate coordination of several key elements of oral homeostasis, namely oral microbiota, saliva, and oral mucosa. This chapter reviews the effects of lactic acid bacteria on common oral diseases
Lactic Acid Bacteria: Microbiological and Functional Aspects

and maintaining oral health. Only approximately 1% of oral cultivable microbiota consists of lactobacilli. These resident bacteria are not known to exert probiotic effects; however, there is evidence to show that external lactic acid bacteria may have beneficial health effects also in the mouth.

The major functions of the mouth relate to the intake of foods and drinks and hence ensuring proper nutrition. Food intake is regulated by complex mechanisms such as taste stimuli, chewing, and lubricating the bolus with saliva to facilitate swallowing. However, in addition, psychic factors play an important role in these basic and life-supporting functions of the mouth. Early in life the mouth is important in organogenesis and later also in the psychic development of a child. It is well known how sensitive the mouth is throughout life. Hence, symptoms and diseases of the oral cavity can have severe consequences and affect well-being in general.

Dental diseases are highly prevalent in all populations. Therefore prevention and treatment of oral and dental diseases have been intensively studied for decades. More recently, potential application of probiotics has also been introduced in this field. The oral microbiota is unique in its microbial contents and variety. The microorganisms are organized in highly specific biofilms. Microbial biofilms are defined as sessile, three-dimensional communities of bacteria irreversibly attached to a substratum, or to an interface, or to each other, respectively, embedded in an exopolysaccharide matrix (Costerton 1995; Costerton et al. 2005; Marsh 2005). Hundreds of bacterial and other microbial species have been identified in samples from the mouth, and modern molecular biological techniques have shown that there are traces of even thousands of different microbial species in the mouth (Aas et al. 2005; Paster et al. 2006; Keijser et al. 2008). Therefore it is particularly challenging to introduce new species such as probiotics in this microcosmos. Common oral diseases in their essence have an infectious etiology and are due to disturbances in the oral microbial homeostasis.

Advances in understanding the composition and morphology of oral biofilms provide information about the spatial and temporal organization of microbial communities, their communication, and their contribution to maintenance of balanced microecology. In this perspective oral biofilms in healthy mouths are populated by species capable of restraining pathogen overgrowth. The idea to use bacteria from normal microbiota to combat harmful bacteria was proposed a century ago (Metchnikoff 1908), and is again gaining popularity.

20.2 Bacterial Composition of the Oral Cavity

Anatomically, the oral cavity is the only part of human body where hard tissues directly penetrate soft tissue epithelium: teeth surrounded by the gingival epithelium and periodontal connective tissue extend from alveolar bone to the oral cavity. When inflamed, the tooth-supporting tissues open the parentral space for the outer environment—consequently, bacteremia of oral origin is relatively prevalent (Bascones-Martínez et al. 2009).

According to recent studies, the resident oral microbiota is indeed composed of numerous species (Keijser et al. 2008). Mouth temperature is optimal for bacteria to grow; there is adequate moisture, frequent serving of nutrients, and a variety of different surfaces for microbial attachment. The epithelium is partly keratinized and partly nonkeratinized depending on the anatomical site in the mouth. Dental hard tissues, enamel, dentin, and root cement offer strong support for biofilm formation, and there are often man-made appliances such as dental restorations and prostheses that further enhance microbial attachment and colonization. A healthy mouth can be colonized by 30–80 of the possible 1000 species at any given time (Aas et al. 2005). The number
of cultivable bacteria in the oral cavity is about $10^{11}$ g$^{-1}$ (wet weight dental plaque) and $10^9$ ml$^{-1}$ saliva (Li et al. 2005). The different oral microenvironments cheek mucosa, palate, tongue, tooth surfaces, gingival margins, and saliva are characterized by stable microbial colonies (Maukonen et al. 2008). Hence, there are major differences in the composition of the microbiota on the different surfaces sampled (Papaioannou et al. 2009). For example, the soft tissue samples are dominated by streptococci, particularly *Streptococcus mitis*, *S. oralis*, and *S. salivarius*, whereas tooth surfaces harbor higher numbers of *Actinomyces* and other species. The *viridans*-group streptococci constitute the majority of the indigenous oral microbiota but practically any human pathogenic bacterium may be encountered in the mouth. Yeasts are also prevalent in particular in elderly individuals. *Candida albicans* is the predominant yeast but recent emphasis has also been focused on non-*C. albicans* yeasts (Meurman et al. 2009). The ability of these species to form biofilms has important clinical consequences due to their increased resistance to antifungal drugs and the ability of yeast cells to withstand host immune defenses within biofilms (Silva et al. 2009).

The microbe-free oral cavity of a newborn begins to acquire a complex consortium of microorganisms soon after birth via saliva-mediated contact with the mother (Ruby and Goldner 2007). The predominant bacterial genus in the normal oral microbiota of a baby born through the vaginal delivery is *Lactobacillus*. However, if the baby is born by Cesarean section, the oral microbiota of the infant develops mainly on feeding. Breast-fed babies acquire gram-positive cocci from the mother’s skin, while bottle-fed babies are predominantly colonized by gram-negative bacilli. Microbial colonization of the mouth is a dynamic process reflecting perpetual interspecies interactions. The structural differences between various oral surfaces predispose the development of specific microbiota in its composition and architecture. Biofilm formation on dental hard and soft tissues is initiated by adsorption of salivary proteins, glycoproteins, and mucins (Marsh and Bradshaw 1995; Wickström et al. 2009a, 2009b; Wickström and Svensäter 2008; Wei et al. 2006). Numerous pellicle proteins such as amylase, lysozyme, or proline-rich proteins have been identified as specific receptors for bacterial adhesion (Hannig et al. 2007). Initially, however, the oral microbiota is relatively simple in composition. Among early oral colonizers are gram-positive streptococcal species such as *S. salivarius* and *S. sanguinis*. Furthermore, Smith et al. (1993) have shown that *S. mitis* constitutes the major component of the initially colonizing streptococcal microbiota of the young infant. The early colonizers are unique in their abilities to coaggregate bacteria of the same genus as well as species of other genera (Kolenbrander et al. 1990, 1993; Egland et al. 2001).

Palmer et al. (2003) were the first to show in vivo that the initial biofilm formation is a result of coaggregation and adhesion between *Streptococcus* species and *Actinomyces* spp. Species of the genus *Fusobacterium*, which are found commonly in both supragingival and subgingival samples, act as bridging microbes between early and late colonizers (Al-Ahmad et al. 2007; Periasamy and Kolenbrander 2010). The dynamics in biofilm development shows that the early colonizers form columnar microcolonies with their long axis perpendicular to the tooth surface, but after 3 weeks these are replaced by filamentous species. This structural organization of the biofilm then remains stable for months. Maturation of the biofilm proceeds via coaggregation of planktonic bacteria to the already adhered biofilm. Moreover bacteria within the biofilm show a pattern of gene expression and protein synthesis that is different from that of their planktonic counterparts (Burne et al. 1997; Motegi et al. 2006). Interactions between the oral microbial residents may influence the properties of the whole community (He et al. 2009). As a whole, within complex biofilms, it is not merely the presence of a single organism, but the interactions between and among biofilm residents that are essential in its function and determine the properties of
a biofilm. Once formed, the composition of biofilm is fairly stable and compatible with health. Indeed, Rasiah et al. (2005) have shown that the predominant oral microbiota can remain stable for 7 years.

### 20.3 Defensive Mechanisms of the Mouth

The most important defense factor in the oral cavity is the oral epithelium, which forms a mechanical barrier and separates the parenteral space from the outer environment. Disruption of the epithelial cell-to-cell contacts may immediately lead to invasion of microorganisms to deeper tissue components and via hematogenic spread all over the body. Keratinization of the epithelium further strengthens the mechanical barrier.

When microbes gain entry in the connective tissue underlying the epithelium, a number of local and systemic defense mechanisms are upregulated against the infection. These include cytokines, which in turn trigger inflammatory reactions with a number of biochemical cascades and subsequent inflammatory cell migration. In later stages of the defensive reactions, specific immune defense is activated. In the oral cavity, however, the systemic defense plays a minor role in comparison to local saliva-mediated systems even though there is interaction between these defensive factors (Janket et al. 2008).

Saliva is the major local defense factor of the mouth (Edgar and O’Mullane 1996). It flushes away microorganisms, which then can be swallowed and subsequently destroyed by gastric acid in the stomach. Normal salivary flow in adults, stimulated by chewing, is approximately 1–2 ml/min while flow rates below 0.7 ml/min are regarded as reduced. For unstimulated resting salivary flow, the respective lower threshold value is 0.1 ml/min. Values below these thresholds indicate clinical hyposalivation, which, however, does not necessarily reflect in subjective dry mouth sensation or xerostomia. Namely, the feeling of how much saliva is enough is highly subjective. The most common reason for hyposalivation is use of systemic drugs. There are hundreds of pharmaceuticals that reduce salivary flow as a side effect (Närhi et al. 1999). Reduced salivary flow directly affects oral microbiota, causing a microbial shift toward colonization of more pathogenic species.

Saliva also contains effective buffering systems, mainly the bicarbonate buffer, for maintaining the neutrality of oral cavity pH. Salivary buffering is essential in protecting the teeth against acid attack caused by fermenting bacteria and/or acidic foods and drinks. Saliva is supersaturated with calcium and phosphate ions, thus providing the key elements for dental hard tissues needed for remineralization in cases with caries.

A variety of specific and nonspecific defensive systems are also functioning in saliva. Secretory immunoglobulins originate from immune cells, which home to the salivary glands and are produced in response to oral microbes. Secretory IgA is the major immunoglobulin, preventing microbial attachment and coaggregation. Saliva also contains lesser amounts of serum-derived IgG and IgM. The immunoglobulins aggregate bacteria and may activate complement system in the gingival crevice but not in saliva (Edgar and O’Mullane 1996).

The important role of the oral cavity defensive systems is particularly seen among medically compromised patients. For example, in immunosuppressed patients undergoing treatment for cancer, the mouth has been shown to be the source of septicemia in 25–45% of the cases, but even extremely high percentages (75%) have been reported in the literature (Meurman et al. 1997), and similarly among organ transplant patients (Rautemaa et al. 2007; Scully 2008). Mouth ulcerations with or without mucosal inflammation or mucositis and gingivitis provide the portals of entry for
infections, emphasizing the importance of intact epithelial cell-to-cell contacts. Bacteremia of oral origin may follow even from normal daily activities such as chewing food (Hartzell et al. 2005). Forner et al. (2006) observed in patients with periodontitis, bacteremia during 30 min after onset of blood sampling at chewing while no bacteria were detected in blood of periodontally healthy subjects. Consequently, periodontal disease has been associated with a variety of systemic diseases where the pathogenic mechanisms involve upregulating inflammatory cytokines and other inflammatory mediators by the bacterial burden (Meurman et al. 2004). Table 20.1 summarizes the salivary defensive systems.

### 20.4 Lactic Acid Bacteria in the Oral Cavity

The lactic acid–producing microorganisms in the oral cavity comprise a large group of species belonging to the genera *Lactobacillus, Lactococcus, Streptococcus, Leuconostoc, Enterococcus*, and *Weissella*. These species contribute substantially to the diversity of commensal microbiota, although lactobacilli only represent about 1% of the cultivable oral microbiota (Marsh 1994). The most commonly isolated lactobacilli from the oral cavity are strains belonging to *L. fermentum* and *L. salivarius* species (Maukonen et al. 2008; Ahrne et al. 1998; Teanpaisan et al. 2006; Köll-Klais et al. 2005; Simark-Mattsson et al. 2007). Table 20.2 demonstrates the diversity of common oral lactobacilli isolated from healthy individuals.

The role of lactic acid bacteria in prevention of common oral diseases has been recently addressed and the list of species with probiotic activity rapidly increases. Lactic acid bacteria with probiotic activity in the oral cavity should possess several distinctive features to be able to contribute to oral health. First of all such species should not impose risk to oral tissues or hamper the key mechanisms of oral homeostasis. Another important aspect of probiotic candidates in the mouth is their ability to establish themselves in oral biofilms, at least temporarily, thus reducing the number of oral pathogens either due to the production of antimicrobial substances, or competition for binding sites or nutrients. To date there are only a few clinical trials advocating the efficiency of some *L. rhamnosus*, *L. reuteri*, *L. casei*, and *W. cibaria* strains that have positively affected some aspects in the development of common oral diseases (Meurman and Stamatova 2007; Stamatova and Meurman 2009).

<table>
<thead>
<tr>
<th>Table 20.1 Salivary Defensive Systems</th>
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<tbody>
<tr>
<td><strong>Saliva Factor or Component</strong></td>
</tr>
<tr>
<td>Flow rate</td>
</tr>
<tr>
<td>Calcium and phosphate</td>
</tr>
<tr>
<td>Buffering</td>
</tr>
<tr>
<td>Mucopolysaccharides</td>
</tr>
<tr>
<td>Proteolytic enzymes, e.g., lysozyme</td>
</tr>
<tr>
<td>Immunoglobulins</td>
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</tbody>
</table>
Dental caries is an infectious disease affecting practically every individual in all countries at some phase of life. However, caries is becoming highly polarized so that a minority of populations has high caries prevalence while the majority is not severely affected. Caries begins as microscopic surface enamel lesion caused by acidogenic bacteria. The process can be reversible if the acidic attack subsides and lesion remineralization takes place from salivary calcium and phosphate. Enamel is almost 100% composed of mineralized apatite crystals with a critical pH of approximately 5.5. Hence, if the pH value drops below this threshold in the microenvironment, apatite dissolution and cavity formation may result. Fluoride is the trace element known to prevent enamel dissolution because fluorapatite is more acid resistant than hydroxyapatite (HA) (Twetman et al. 2003, 2004). Thus, fluorides are used for caries prevention and in enhancing enamel remineralization. Macroscopic lesions (i.e., cavities) are beyond reparative functions, however. If the pathologic caries process continues, then dentin becomes affected and, finally, dental pulp will be infected. Pulp necrosis may follow with subsequent spread of the infection through the apical foramen of the tooth into jaw bones, leading eventually to systemic hematogenic spread of the infection.

Dental caries is the result of the complex interaction between dietary carbohydrates and cariogenic microorganisms in oral biofilms, also influenced by the quality and quantity of saliva; the disease results from shifts in the balance of the resident microbiota driven by changes in the local environmental conditions (Aas et al. 2008). Bacteria associated with caries belong to the normal microbiota of the oral cavity and dental caries is therefore an endogenous infection (Nyvad and Fejerskov 1989, 1990; Takahashi and Nyvad 2008).

### Table 20.2 Common Lactobacilli Species Isolated from the Oral Cavity

<table>
<thead>
<tr>
<th>Lactobacillus Species</th>
<th>Study Group</th>
<th>Oral Site</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>L. salivarius</td>
<td>Teenage orthodontic patients</td>
<td>Tooth surface, plaque</td>
<td>Botha 1993</td>
</tr>
<tr>
<td>L. casei</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L. fermentum</td>
<td>44 healthy subjects</td>
<td>Teeth, tongue, saliva, gum</td>
<td>Colloca et al. 2000</td>
</tr>
<tr>
<td>L. plantarum</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>L. salivarius</td>
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<td></td>
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<tr>
<td>L. rhamnosus</td>
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<tr>
<td>L. gasseri</td>
<td>15 healthy adults</td>
<td>Saliva, subgingival sites</td>
<td>KölI-Klais et al. 2005</td>
</tr>
<tr>
<td>L. fermentum</td>
<td></td>
<td></td>
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<tr>
<td>L. s.</td>
<td>16 adults</td>
<td>Saliva</td>
<td>Hojo et al. 2007</td>
</tr>
<tr>
<td>L. gasseri</td>
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<tr>
<td>L. fermentum</td>
<td></td>
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<td></td>
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<tr>
<td>L. s.</td>
<td>14 healthy adults</td>
<td>Tooth, oral mucosa</td>
<td>Strahinic et al. 2007</td>
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<tr>
<td>L. fermentum</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>L. plantarum</td>
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<td></td>
<td></td>
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<tr>
<td>L. salivarius</td>
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<td></td>
<td></td>
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<tr>
<td>L. paracasei</td>
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Caries infection is predominantly transmitted by *S. mutans*, a gram-positive bacterium belonging to the *viridians* group (Newbrun 1992). *S. mutans* is able to live in the biofilm on dental enamel with help of highly developed extracellular polysaccharide metabolism. The nature and consistency of dietary carbohydrates is also important, and sticky, toffee-like foodstuffs are more detrimental to teeth than liquids because of their longer clearance time in the oral cavity. Sucrose is more cariogenic than lactose, for example, because sucrose is more rapidly metabolized by the streptococci.

However, recent studies have shown that caries is a result of synergistic activity of several species not solely *S. mutans*. No detectable levels of these species were reported in 10–15% of caries-active subjects, thus indicating that the presence of *S. mutans* does not necessarily reflect caries activity (Beighton 2005). A wide group of microorganisms are in fact identified from carious lesions. Of these, *S. mutans*, *L. acidophilus*, and *Actinomyces viscosus* may be considered the main pathogenic species involved in the initiation and development of dental caries (Shivakumar et al. 2009).

The role of some lactic acid bacteria to reduce the risk of caries development has been advocated in several studies. For example, *L. rhamnosus* GG given in milk to preschool children for 7 months was able to reduce caries risk (Näsé et al. 2001). *L. reuteri* and *L. casei* were also able to affect the number of cariogenic streptococci in the mouth (Çaglar et al. 2006, 2007, 2008; Ahola et al. 2002; Stecksén-Blicks et al. 2009). However, in all these studies, lactobacilli were administered intentionally in the oral cavity. If the probiotic species were permanent residents in the mouth, they might nevertheless be more beneficial from the dental caries point of view because the bacteria might then better interact with pathogenic species and contribute to a healthy state. It is probable that in order to achieve this goal, however, lactobacilli should become incorporated in oral biofilms at early infancy. Thus far only one study has shown that this might be possible (Yli-Knuuttila et al. 2006).

Lactobacilli habitually harboring the mouth have shown various degrees in inhibiting growth of common caries pathogens. Simark-Mattsson et al. (2007) have shown that *L. plantarum*, *L. paracasei*, and *L. rhamnosus* most frequently isolated from caries-free mouths were strong inhibitors of *S. mutans* and *S. sobrinus*, both reference and clinical isolates. This study also demonstrated that the lactobacilli composition is different in healthy and caries-active mouths. Hence, lactobacilli with pathogen-inhibiting properties may naturally colonize the oral cavity and suppress the growth of some oral pathogens. Plausible explanation to the observed phenomena could be the ability of those species to secrete inhibitory substances and/or to compete for binding sites in oral biofilms, which subsequently reduces the levels of caries pathogens. *In vitro* tests have revealed that oral lactobacilli can indeed inhibit the growth of *S. mutans* in a species- and strain-dependent manner (Köll-Klais et al. 2005, 2008; Strahinic et al. 2007). Antimicrobial activity is supposed to be related to bacteriocin production or to the production of antimicrobial substances of nonproteinaceous nature, such as lactic acid or acetic acid (Strahinic et al. 2007).

The specific coaggregation of mutans streptococci with lactobacilli can also be regarded as a defensive mechanism in caries prevention. Lang et al. (2010) have found that among 624 lactobacilli strains tested, only four *L. casei susp. paracasei* and two *L. rhamnosus* strains coaggregated with *S. mutans*. The observed coaggregation properties were heat stable and not affected by protease treatment, which allows some of these lactobacilli strains to be considered for future oral applications. Reduction of cariogenic species in oral biofilms includes competitive exclusion, displacement of pathogens, production of antibacterial compounds, and the release of biosurfactants. In a dual-species biofilm model, *S. salivarius* produced proteinaceous substances that could effectively inhibit biofilm formation by *S. mutans* (Tamura et al. 2009). *L. rhamnosus* and *L. casei*...
Shirota adhering to saliva-coated HA have been shown to diminish the adherence of *S. mutans* when they were allowed to adhere before the streptococci (Haukioja et al. 2008). However, after streptococcal pretreatment of saliva-coated surfaces only *L. casei* ATCC 11578 retained the capability to slightly inhibit the adherence of *S. mutans* and even to release the already bound streptococci from the HA. Figure 20.1 shows the variation in adhesive properties of several common probiotic lactobacilli to saliva-coated HA.

The ability of lactic acid bacteria with probiotic properties to integrate in oral biofilms can efficiently modulate the pathogenic load in these microbial ecosystems. *L. rhamnosus* GG and *L. plantarum* alone are forming pallid, thin biofilms, whereas when present with *A. naeslundii* or *A. gerencseriae* they are able to proliferate and accrue in a large biofilm with higher cell density, indicating that there may be potential for modulating the biofilm composition by targeting specific partner species (Filoche et al. 2004). Furthermore, some probiotic lactic acid bacteria can modify the composition of salivary pellicle, thus altering its microbial binding preferences. For example, *L. reuteri* SD2112 and *Lactococcus lactis* can produce a significant reduction in salivary peroxidase and agglutinin pg340 in newly formed salivary pellicle due to binding or degradation of the above proteins (Haukioja et al. 2008).

Although there are several clinical trials substantiating the effect of probiotic lactic acid bacteria in caries risk reduction, sugar metabolism of these species should not expose a risk to dental hard tissues. Namely, several lactobacilli species are regarded as caries pathogens. Studies in rodents with *L. casei* (Rosen 1969; Rosen et al. 1968), *L. acidophilus* (Fitzgerald 1968), *L. salivarius* (Matsumoto et al. 2005), and other species of lactobacilli (Fitzgerald et al. 1980) have shown that lactobacilli indeed contribute to caries development. Conversely, investigations by Michalek et al. (1981) supported the idea that lactobacilli have less affinity for the tooth surface of gnotobiotic rats than do *S. mutans*. It was observed that these two genera of oral bacteria have completely different

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**Figure 20.1** Adhesion of common probiotic lactobacilli to saliva-coated HA (saliva), and bovine albumin pretreated saliva-coated HA (APS). (Modified from Haukioja, A. et al., *Oral Microbiol. Immunol.* 23, 336–343, 2008.)
sites and ecological determinants for colonization in this animal model. The relatively low number of lactobacilli isolated from saliva, less than 1%, can therefore be considered insubstantial for a pronounced cariogenic activity.

Although the role of lactobacilli in caries development is ambiguous, they may contribute to caries progression in cases of established caries lesions. In an artificial caries model, Lima et al. (2005) have shown interspecies difference in tropism to carious dentin when comparing \textit{L. casei} Shirota with \textit{L. acidophilus}. It was verified that a lower number of \textit{L. casei} Shirota was attached to carious dentin when compared with \textit{L. acidophilus}. Additionally, when evaluating saliva and biofilm samples of individuals consuming daily 200 ml of yogurt with \textit{L. casei} and \textit{L. acidophilus}, Busscher et al. (1999) observed that these microorganisms could be found in the biofilm samples.

Nevertheless, aciduric and acidogenic properties of most lactic acid bacteria merit special concern with respect to caries. Bacteria differ in their metabolic capabilities and metabolic end products may either serve as growth substrates or inhibit the growth of other species. The \textit{Lactobacillus} group contains homofermentative and heterofermentative species but they all are aciduric. With the increasing number of probiotic species suggested for oral health maintenance, the assessment of their fermentation profiles is essential for ruling out potential deleterious effects on dental tissues. As said, common probiotic and dairy lactobacilli can produce acids efficiently from glucose and thus lower the pH to different degree in a strain- and species-dependent manner (Haukioja et al. 2008). Sucrose, fructose, and glucose are considered the most important carbohydrates involved in the caries process (Rölla 1989; Mundorff-Shrestha et al. 1994; Lingström et al. 2000) and they are fermented differently by various strains. Among probiotic lactobacilli, \textit{L. plantarum} strains can generally rapidly metabolize a wide range of dietary sugars, whereas \textit{L. paracasei}, \textit{L. reuteri}, and \textit{L. rhamnosus} seem to be fairly harmless in this regard (Hedberg et al. 2008). Figure 20.2 demonstrates growth pattern of \textit{L. rhamnosus} GG when grown in different sugar-containing culture media.

In general, use of probiotics to combat dental caries seems a promising approach. However, more controlled studies are called for further evidence, and it appears that the selection of proper probiotic species is essential also in the safe-for-teeth perspective. Table 20.3 summarizes results from clinical studies on dental caries where probiotics have been used.

![Figure 20.2 L. rhamnosus GG growth curve in different sugar-containing media. OD, optical density.](image-url)
Periodontal disease affects approximately 25–35% of the adult populations; the rate varies across geographical regions. The disease may take decades to manifest but also a juvenile form is known affecting young people. Periodontal disease is an infectious and inflammatory disease caused by chronic exposure of tooth-supporting tissues to microorganisms of the oral biofilm. Anaerobic bacteria such as Porphyromonas gingivalis, Tannerella forsythia, Aggregatibacter actinomycetemcomitans, and Treponema denticola are considered periopathogens; that is, these bacteria have been associated with the prevalence and progression of the disease. The mere existence of dental plaque and particularly calcified plaque or dental calculus triggers inflammatory reactions in periodontal tissue, leading to tissue damage and loss of tooth support. Ultimately periodontal disease leads to loss of teeth after which the inflammation subsides (Pihlstrom et al. 2005).

The composition of lactobacilli species differs in healthy and periodontitis patients and obligate homofermentative bacteria are less prevalent in chronic periodontitis (Kõll-Klais et al. 2005). Oral intake of some lactobacilli on the other hand can promote reduction of gingival inflammation (Twetman et al. 2009). A 14-day administration of L. reuteri was sufficient to reduce clinical signs in patients with moderate to severe gingivitis (Krasse et al. 2006). Another species, L. salivarius WB21, has also been shown to positively affect periodontal health. Concentrations of salivary inflammatory markers of periodontal disease appeared to decrease in smokers after using L. salivarius WB21 tablets for 8 weeks (Shimauchi et al. 2008). Periodontal inflammation was also found to be reduced after the intake of probiotic tablets (Bifidobacterin and Acilact) available on the Russian market (Grudianov et al. 2002). Studies from Russia have further shown that a periodontal dressing containing L. casei 37 reduced the number of most common periodontal pathogens and extended remission up to 10–12 months (Volozhin et al. 2004). A possible explanation to the results might be the inhibitory effect of lactobacilli on pathogen growth, thus altering the composition of supra- and subgingival biofilm. Due to its ability to inhibit P. gingivalis, L. salivarius TI 2711 was given for 4 or 8 weeks in a tablet to healthy volunteers at a concentration of $2 \times 10^7$ CFU/ml. A significant reduction of black-pigmented rods in saliva was observed, which gives promise to periodontal use of probiotics (Ishikawa et al. 2003).

### Table 20.3 Clinical Studies on Dental Caries Where Probiotics Have Been Used

<table>
<thead>
<tr>
<th>Lactobacillus Species</th>
<th>Vehicle of Administration</th>
<th>Duration</th>
<th>Observed Effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>L. rhamnosus GG</td>
<td>Cheese</td>
<td>3 weeks</td>
<td>Reduction of S. mutans</td>
<td>Ahola et al. 2002</td>
</tr>
<tr>
<td>L. rhamnosus GG</td>
<td>Milk</td>
<td>7 months</td>
<td>Lower S. mutans counts</td>
<td>Näse et al. 2001</td>
</tr>
<tr>
<td>Bifidobacterium DN-173010</td>
<td>Yogurt</td>
<td>4 weeks</td>
<td>Reduction of S. mutans</td>
<td>Çaglar et al. 2005</td>
</tr>
<tr>
<td>B. animalis subsp. lactis DN-173010</td>
<td>Fruit yogurt</td>
<td>4 weeks</td>
<td>Reduction of S. mutans</td>
<td>Cildir et al. 2009</td>
</tr>
</tbody>
</table>

### 20.6 Periodontal Disease

Periodontal disease affects approximately 25–35% of the adult populations; the rate varies across geographical regions. The disease may take decades to manifest but also a juvenile form is known affecting young people. Periodontal disease is an infectious and inflammatory disease caused by chronic exposure of tooth-supporting tissues to microorganisms of the oral biofilm. Anaerobic bacteria such as Porphyromonas gingivalis, Tannerella forsythia, Aggregatibacter actinomycetemcomitans, and Treponema denticola are considered periopathogens; that is, these bacteria have been associated with the prevalence and progression of the disease. The mere existence of dental plaque and particularly calcified plaque or dental calculus triggers inflammatory reactions in periodontal tissue, leading to tissue damage and loss of tooth support. Ultimately periodontal disease leads to loss of teeth after which the inflammation subsides (Pihlstrom et al. 2005).

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In vitro studies have shown that different lactic acid bacteria can inhibit growth of some common microorganisms associated with periodontal diseases. Both homo- and heterofermentative oral lactobacilli can suppress the growth of periodontal pathogens. L. paracasei, L. plantarum, L. rhamnosus, and L. salivarius can substantially inhibit the periodontal bacteria A. actinomycetemcomitans, P. gingivalis, and P. intermedia (Köll-Klais et al. 2005; Stamatova et al. 2007). Figure 20.3 illustrates growth inhibition of some common periodontal pathogens by lactobacilli.

Regular mouth rinsing with a product containing substances secreted by Bacillus subtilis in its culture medium could significantly improve periodontal health by decreasing periodontal index scores and reducing the number of harmful periodontal pathogens (Tsubura et al. 2009). It is plausible that such a product may modify oral microbiota, activate gingival fibroblast cells, and induce cytokine production. Milk as a means for administrating L. casei Shirota has also been beneficial in improving periodontal health. An 8-week intake of a probiotic milk drink reduced elastase activity and the levels of matrixmetalloproteinase-3 in gingival crevicular fluid (Staab et al. 2009).

Oral administration of a probiotic tablet containing L. salivarius WB21 could significantly reduce the counts of several periodontal pathogens, A. actinomycetemcomitans, P. intermedia, P. gingivalis, T. denticola, and T. forsythia, in subgingival dental plaque after 4 weeks of probiotic intervention (Mayanagi et al. 2009). The exact mechanisms by which probiotic bacteria reduce pathogen levels still remain unclear. However, the promising results from the first clinical studies cited above further support the idea that probiotic applications might be recommended for the prevention and treatment of periodontal disease in the future.

20.7 Yeast Infections

Oral mucosal infections are mainly caused by C. albicans and the frequency increases with age (Richardson and Warnock 2003). Up to 88% of diseased elderly have been reported to carry Candida sp. in the mouth (Wilkeson et al. 1991). Concomitant use of several drugs, particularly antimicrobial agents, causes selective suppression of resident bacteria in the oral microbiota leading to yeast overgrowth. Wearing dentures has long been known to contribute to yeast infection, and good oral and denture hygiene has been shown to decrease the colonization of Candida (Grimoud et al. 2005). Candida may also invade in deeper tissues, but luckily systemic yeast
infections are rare and mainly encountered in high-risk patients. However, mortality in systemic *Candida* is of the magnitude of 40%. These potentially fatal infections are seen in intensive care units and in patients with severe and prolonged neutropenia, and often with multiorgan failure (Richardson and Warnock 2003).

Yeast infections are treated with special antifungal drugs such as broad-spectrum azoles, liposomal amphotericin B, and the newer echinocandin agents with improved efficacy and tolerability profiles (Gullo 2009). Although treatment outcome is favorable in most cases, preventive measures for oral candidiasis are definitely more desirable than the frequent use of antifungals that may lead to the development of drug resistance. In this perspective, the potential of probiotics needs to be investigated.

The first studies providing evidence for the positive effect of lactic acid bacteria on severity and duration of *Candida* infection were conducted a decade ago on mice. Wagner et al. (2000) have shown that immunodeficient mice fed with heat-killed *L. acidophilus* NCFM and *L. rhamnosus* GG had less severe manifestations of orogastric candidiasis when compared with control animals. Dissemination and viability of *C. albicans* was inhibited after lactobacilli administration, thus highlighting the protective role of probiotic candidates on oral yeast infections. Enhanced clearance of *C. albicans* from the oral cavity of mice has also been achieved after *L. acidophilus* LAFTI L10 administration (Elahi et al. 2005). After 6 days of *L. acidophilus* administration, no detectable levels of yeasts were observed in the test group animals and the protective effect pertained for 2 weeks after cessation of oral feeding. It has been observed that the accelerated clearance of *C. albicans* from the oral cavity correlated with an early appearance of mRNA for interleukin-4 and interferon-γ (Elahi et al. 2005).

There is only one clinical study on humans assessing the effect of oral intake of probiotic lactobacilli on *Candida* infection in elderly. Hatakka et al. (2007) observed a 32% reduction in the prevalence of subjects with high (≥10⁴ CFU/ml) salivary yeast counts after 16-week probiotic cheese intake (containing *L. rhamnosus* GG, *L. rhamnosus* LC705, and *Propionibacterium freudenreichii* ssp. *shermanii* JS) in a randomized, double-blind, placebo-controlled study on 276 elderly patients. The anticipated mode of probiotic activity against *Candida* infections is immunomodulation of host response to the yeast challenge.

It has been shown that *Candida* species can degrade structural basement membrane components such as human laminin-332, fibronectin, and E-cadherin in a strain-dependent manner, thus weakening host tissue defense mechanisms (Pärnänen 2010). On the other hand, *in vitro* studies with probiotic lactobacilli in the gastrointestinal tract have shown that probiotic-epithelial cell cross-talk may strengthen the epithelial tight junctions that increases resistance to pathogen invasion (Otte and Podolsky 2004; O’Hara et al. 2006; Zyrek et al. 2007). In this perspective further *in vitro* studies are required to address the molecular mechanisms of probiotics—*Candida*—host interactions in the oral cavity. Lactobacilli isolated from the oral cavity have shown no ability to inhibit *C. albicans* growth, suggesting that the probable positive effect of probiotic species is indeed due to active microbe—host epithelium cross-talk (Köll et al. 2008). In general, more studies are needed for stronger scientific evidence in the area of probiotics vs. oral yeast infections.

### 20.8 Nonspecific Symptoms of the Mouth

Xerostomia or subjective feeling of dry mouth is highly prevalent in elderly populations, ranging from 10% to 40% (Nedefors 2000). Medications are responsible for a significant proportion of cases with xerostomia and the list of drugs that affect saliva secretion includes more than 400...
agents (Närhi et al. 1999). Several systemic diseases also affect salivary secretion. For example diabetes mellitus is clinically manifested with hyposalivation and dry mouth, and subsequently with an altered oral microbial balance. Patients with hyposalivation demonstrate significantly higher numbers of mutans streptococci, Lactobacillus species, and Candida spp. in their saliva (Khovidhunkit et al. 2009). In this context, it must be emphasized that subjective xerostomia does not necessarily reflect in reduced salivary flow rates because the feeling of “how much saliva is enough” is highly subjective.

Several studies indicate that the risk of xerostomia increases with the increasing use of medications. Reduced saliva flow inevitably also reduces defensive mechanisms in the oral cavity. Consequently, dry mouth should be treated. However, apart from drinking water, using local oral gels and other preparations for dry mouth, pharmacological means for ameliorating xerostomia are few. Pilocarpine (5 mg tablets taken several times daily) has been used in cases with severe hyposalivation if no systemic contraindications exist for using the cholinergic drug.

There is only one clinical study published to date providing weak evidence of the positive effect of L. rhamnosus GG given in cheese. The intervention appeared to improve symptoms of hyposalivation in the elderly subjects investigated (Hatakka et al. 2007). Consequently, further systematic studies with lactic bacteria are needed to clarify the role of probiotics on hyposalivation and xerostomia. Theoretically, the salivary flow-improving effect might be mediated through enhanced immune function and overall reduction in systemic and local inflammatory state. However, as said, studies are needed to confirm this hypothesis.

20.9 Halitosis

Halitosis, foetor ex ore, oral malodor or bad breath, is a condition essentially ascribed to the production of volatile sulfur compounds (VSC). They are mainly dihydrogen sulfide and methyl mercaptan generated by gram-negative anaerobes residing in periodontal pockets and on dorsum of the tongue. Nasal, throat, laryngeal, and gastrointestinal problems can also cause bad breath and a number of systemic diseases are characterized with often specific odor. These include, for example, diabetic ketoacidosis and uremia.

The prevalence of halitosis is not known. Based on clinical experience, however, it may be assumed that the nuisance is highly common. It has been estimated that in up to 40% of cases without underlying organic disease the reason is in the mouth. Therefore good professional oral health care combined with a daily regimen of oral hygiene, including tooth and tongue cleaning, and optimal use of mouth rinse solutions may be of help (Hughes and McNab 2008).

Use of lactic acid bacteria to treat oral malodor has also been introduced recently. S. salivarius K12 taken in a lozenge was found to reduce the levels of VSC in 85% of the subjects 1 week after administration (Burton et al. 2006). S. salivarius K12 possessed pronounced inhibitory activity against black-pigmented species contributing to halitosis. The regular intake of the streptococcal strain could eventually modify the composition of oral microbiota, thus reducing the number of odor-producing microorganisms. S. salivarius K12 may temporarily colonize the oral cavity with pronounced tropism to mucosal membranes (Horz et al. 2007).

W. cibaria, a lactic acid bacterium, formerly included in the genus Lactobacillus, and which has never been associated with pathogenicity, has also shown promising in vivo results in the treatment of halitosis. Substances generated from W. cibaria isolates could exert reducing effects on VSC production by inhibiting F. nucleatum proliferation (Kang et al. 2006). In general, however, for true evidence this area also calls for further studies.
20.10 Conclusion

Apart from some promising clinical results of probiotic lactic acid bacteria in the prevention and control of dental caries, the complex oral microbiota is a big challenge for combating oral and dental diseases. Introducing external microorganisms with proper effect on the, by nature, stable biosystem of the mouth is not an easy task to perform. There are many lactic acid bacteria as residents of the normal oral microbiota. However, many of these bacteria are potentially harmful to teeth due to their acid fermentation capacity. Occasionally, also known probiotic bacterial species can be detected in mouth samples. It is questionable, however, whether permanent colonization takes place in established oral biofilms. Nevertheless, if exogenous beneficial bacteria are introduced at the development phase of oral biofilm, that is, in early infancy, there is a possibility that these microbes become permanently colonized. Because the infectious oral and dental diseases are highly prevalent in all populations, the probiotic approach in controlling these diseases indeed is interesting and warrants further studies.

Acknowledgments

Supported by the Helsinki University Central Hospital (EVO Grant TYH3245).

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Some Considerations for the Safety of Novel Probiotic Bacteria

Diana C. Donohue and Miguel Gueimonde

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21.1 Probiotics Past and Future

Maintenance of normal intestinal microbiota and improved digestion are benefits generally ascribed to existing probiotics. Different bacterial strains are being investigated for their therapeutic potential to treat inflammatory bowel disease, inhibit pathogenic bacteria, ameliorate diarrhea of various etiologies, prevent dental caries, and reduce allergy. It is postulated that probiotic bacteria also have a role in immune modulation and suppression of carcinogenesis. While research has mainly concentrated on probiotics to modulate intestinal microbiota, studies have also sought to restore the vaginal and urinary ecology (Reid et al. 2001).
Probiotic organisms are commonly from the genera *Lactobacillus* and *Bifidobacterium*, strains of *Enterococcus* and *Saccharomyces* species being among the exceptions. Members of these genera are generally regarded as safe because they have a long history of use. They have been associated with disease only rarely, usually as opportunistic infections in people with predisposing conditions (Donohue et al. 1998; Ouwehand and Salminen 2003; Lahtinen et al. 2009). A recent study, conducted with the probiotic strain *Escherichia coli* Nissle 1917, has shown that host-related factors may play a key role on the safety of probiotic strains (Gronbach et al. 2010).

Many of the organisms to which we ascribe probiotic effects have had their origins in fermented foods or the human gut microbiota. Thus they have been consumed with food, or have been in close contact with humans, for centuries with no apparent adverse effects.

Several organizations have addressed the safety of probiotics issue. In the United States most probiotics consumed in foods and dietary supplements are accorded a generally recognized as safe (GRAS) status. The International Dairy Federation and the European Food and Feed Cultures Association have jointly produced a referenced inventory of microorganisms with a documented history of safe use in food manufacture (Mogensen et al. 2002a; Mogensen et al. 2002b). The inventory lists taxonomy and applications for lactic acid bacteria (LAB), *Enterococcus* and *Streptococcus* species, yeasts, and molds. It is an evolving document that will be modified over time. This initiative is a systematic attempt to catalogue organisms that may reasonably be expected to be safe because of their consumption in foods without apparent ill effect. It provides a basis for genera, species, and strains to be identified as safe and a potential source of new probiotic organisms. More recently the European Food Safety Agency (EFSA) has included most of the commonly used microorganisms in the Qualified Presumption of Safety (QPS) list (EFSA 2007). The QPS approach establishes the safety aspects that should be determined and fulfilled for a certain taxonomic unit. According to this approach a probiotic could be given QPS status, QPS with qualifications that requires assessing some specific features of the microorganisms related to its safety, or “not suitable for QPS,” in which case a full safety assessment would be needed.

Nevertheless, our current knowledge applies only to known probiotics already in use. However, new species and more specific strains of probiotic bacteria are constantly being sought for novel probiotic products. The safety status of novel organisms intended for probiotic use cannot be assumed. Before the incorporation of novel strains into products, their efficacy should be carefully assessed and an evaluation made as to whether they share the safety status of traditional food-grade organisms. This process of safety assessment of new strains requires proper strain identification as the key starting point.

The concept of genetic manipulation of bacteria for a specific probiotic function is appealing. Consumer resistance to genetically modified organisms (GMO) in foods is such that GMO probiotics are unlikely in the near future, with the possible exception of clinical applications. Steidler et al. (2000) has shown that a probiotic *Lactococcus lactis* genetically engineered to secrete cytokine interleukin-10 prevented colitis in a mouse model. A recombinant strain of *Bacillus subtilis* 2335 has been designed, which produces proteins with antibacterial and antiviral properties shown to enhance the effectiveness of antitumor therapy in mice (Kaur et al. 2002 and references therein). Probiotics can thus be designed to produce potent bioactive chemicals. Extrapolation from proof of principle in a murine model to development of therapeutic applications for humans demands a stringent and specific safety assessment of such GMO probiotics.

The demonstration of efficacy in probiotics offers vast opportunities for the development of human and veterinary products. The addition of novel bacterial strains to foods and therapeutic products requires reconsideration of the procedures for safety assessment. Probiotic products,
which claim specific nutritional, functional, or therapeutic characteristics, blur the boundaries between what is a food, a diet supplement, or a medicine, posing challenges for regulators.

Evidence for the safety and efficacy of probiotic organisms has until recently been largely anecdotal or based on relatively little, and often poorly designed research. LAB and yeasts intrinsic to the production of traditional foods have been accepted as safe without any real scientific criteria, partly because they exist as normal commensal microbiota and because of their presence for generations presumably without adverse effect.

The introduction of a new probiotic culture demands that it be at least as safe as its conventional counterparts. Suggested safety criteria have included but not been limited to unequivocal identification of species and strain, with candidate strains lodged in culture collections for reference and comparison; a profile of intrinsic properties of the organism such as metabolic and enzyme activities, antibiotic resistance, and the potential for its transference; host-specific behavior of the strain; and host factors predisposing to infection (Salminen et al. 1998; Ouwehand and Salminen 2003). Another issue that deserves more attention is the long-term effect of the use of probiotics, especially in those cases in which the microbiota composition or the immune responsiveness have been modulated.

21.2 Unequivocal Identification

The taxonomy of many probiotic microorganisms has changed significantly with the advent of genetic methods of classification. Strains previously thought to be dissimilar have merged, while other strains have been added or reassigned to different species. This has increased confusion and hindered the proper identification of probiotic strains. However, the persistent use of incorrect or nonexistent species names on product labels despite taxonomic reassignment is an issue for the safety and credibility of probiotics and, therefore, it is not acceptable. Inaccurate nomenclature has no scientific or regulatory validity, misinforms or confuses the consumer, and compromises the safety of the product.

For many consumers the term probiotic is a new concept and they are reliant on the manufacturer's label for appropriate information. The consumer is entitled to expect that the label on a probiotic product accurately reflects its contents: the organism is what it purports to be, it is present alive in a specified concentration range for a stated period, and the suggested serving size contains sufficient organisms to achieve the claimed benefit.

Most of the currently used probiotics belong to the genera *Lactobacillus* and *Bifidobacterium*, which are two genera of gram-positive, non-spore-forming microorganisms. Lactobacilli are generally aerotolerant while bifidobacteria are anaerobes. The genus *Bifidobacterium* shares phenotypic features and habitat with many lactobacilli and other LAB. However, they are phylogenetically distinct, with bifidobacteria having DNA with high guanine and cytosine (G+C) content (55–67%) and belonging to the phylum *Actinobacteria*, while LAB form part of the so-called *Clostridium* branch of the phylum *Firmicutes*, and are characterized by a low G+C content. In addition, bifidobacteria possesses a particular metabolic pathway for hexose fermentation, characterized by the fructose-6-phosphate phosphoketolase (F6PPK) enzyme activity.

The safety of a putative novel probiotic strain is contingent on its accurate identification. Is the strain associated with safe food use, an intestinal strain isolated from humans, a strain isolated from animals, or a genetically modified strain? As probiotic effects are known to be strain specific, it is essential for unequivocal identification of probiotic bacteria at the genus, species, and strain level. The QPS approach, recently established by the EFSA, considers proper identification of the
strain as the first pillar for safety assessment (EFSA 2007). Moreover, correct taxonomic identification of both species and strain is a safety issue also for quality control of the product, consumer or prescriber information, diagnosis and appropriate treatment of suspected clinical cases, and epidemiological surveillance of the exposed population.

Combinations of phenotypic and molecular techniques are available to identify species and discriminate between strains. Closely related strains may be differentiated by molecular typing techniques such as DNA fingerprinting by pulsed field gel electrophoresis, random amplified polymorphic DNA, ribotyping, restriction enzyme analysis, and plasmid profiling. The Food and Agriculture Organization–World Health Organization (FAO-WHO 2006) expert group recommends that phenotypic tests should be done first, followed by genetic identification, using methods such as DNA–DNA hybridization, 16S RNA sequencing, or other well-established methods. In this respect, the European Union (EU)-funded project PROSAFE concluded that biochemical tests should not be used as a stand-alone approach. The use of 16S rRNA gene sequence analysis was considered the best tool for routine determinations. Moreover, the use of sequence-based methods was encouraged given the high reproducibility and data exchangeability of these techniques (Vankerckhoven et al. 2008). The current availability of molecular methods makes the improper identification and labeling of probiotics unacceptable as failure to properly identify the strains may lead to the inclusion of potentially harmful microorganism in the food chain (Yeung et al. 2002; Fasoli et al. 2003).

Special attention should be paid to strain identification, as it has been reported that the identity of microorganisms in probiotic products often does not correspond to the information stated on the product label (Yeung et al. 2002; Fasoli et al. 2003; Temmerman et al. 2003; Masco et al. 2005; Theunissen et al. 2005). Yeung et al. (2002) used partial 16S rDNA sequencing to identify strains obtained directly from the manufacturer and found discrepancies in species designations for almost 50% of the cases. Weese (2002) identified isolates from eight veterinary and five human probiotics to find accurate descriptions of organisms and concentrations for only 2 of 13 products. Temmerman et al. (2003) found that of isolates from 55 European probiotic products, 47% of food supplements and 40% of dairy products were mislabeled. An EU-funded project showed that 28% of the commercial probiotic cultures are misidentified at present by their manufacturers or distributors, which may explain the disagreements observed between the label information and the true identity of the isolated microorganisms in many products (Huys et al. 2006). These studies corroborate previous findings and demonstrate continued inaccurate identification and mislabeling of probiotic products.

In the near future the increasing availability of whole genomes and the affordable price of genome sequencing will allow genome-wide and/or multilocus phylogenetic analysis (Ventura et al. 2006), which is likely to become the standard for identification of probiotic strains of commercial use.

In conclusion, it is clear that strains used by the food industry and scientists should be properly identified using appropriate molecular methods and up-to-date taxonomical nomenclature. It is also important to make all study strains available in international culture collections. Even nowadays, many scientific articles are published without reporting data on the strains used, which is limiting the progress of scientific development in this area.

### 21.3 Enterococci as Probiotics

Probiotics are not limited to lactobacilli or bifidobacteria. Other LAB groups or other food-associated genera have also been used, including enterococci. The assessment of the safety of
enterococci is complicated, even though select strains have been used in probiotic products for some time. Enterococci are commensal microflora of the gastrointestinal tract, occur naturally in some foods, and are common in veterinary probiotics. Several species are pathogens and have been isolated in nosocomial and other infections, often in pure culture. The genus *Enterococcus* rank second or third in frequency of bacteria isolated from hospitalized patients and some virulence factors have been identified (Kayser 2003). The incidence of those virulence determinants among food isolates studied thus far appear to be strain specific (Franz et al. 2003). Moreover, some enterococci demonstrate resistance to antibiotics, including vancomycin, and have the ability to transfer antibiotic resistances (Ouwehand and Salminen 2003). Therefore, lack of virulence traits and transferable antibiotic resistance genes, in any specific strain, should be checked as part of the safety assessment of probiotic enterococci.

Lund et al. (2002) evaluated the ability of viable *Enterococcus faecium* to survive gastrointestinal transit in a prospective, randomized blind study. Volunteers consumed 150 ml daily for 10 days of fermented milk product containing Causidio® culture, a mixture of two strains of *Streptococcus thermophilus* and one of *E. faecium* (4.5 × 10⁹ to 7.5 × 10⁹ colony-forming units of *E. faecium*). Half of the subjects were treated simultaneously with vancomycin (12 mg four times daily). In subjects given probiotic alone, the percentage of probiotic *E. faecium* recovered from feces at day 10 of intake compared with total *E. faecium* ranged from 100% (three subjects) to 13–52% (three subjects) and <2% (four subjects). The strain was not detected 3 weeks after intake ceased, suggesting it does not persist. Probiotic *E. faecium* was not detected at day 10 in vancomycin-treated subjects, probably because this strain is vancomycin sensitive and colonization was prevented. For some subjects the probiotic was the predominant strain. This strain has previously been shown by the authors to acquire the vanA cluster gene for vancomycin resistance *in vitro* (Lund and Edlund 2001). Therefore, it would be prudent to consider *in vivo* conjugation in a safety evaluation.

The increasing importance of *Enterococcus* as a nosocomial opportunistic pathogen, its intrinsic capacity for survival in the human gut and the pathogenicity of some species, coupled with their tendency to exchange genetic material and acquire antibiotic resistance determinants, renders their use as probiotics questionable.

### 21.4 Spore-Forming Probiotics

Other genera such as *Clostridium* and *Bacillus*, which include opportunistic pathogens and/or toxin producers such as *B. cereus* or *B. subtilis*, have also been used as probiotics (Ouwehand and Salminen 2003).

*Bacillus* species are spore-forming bacteria, ubiquitous in the environment and considered to have low pathogenicity. *B. clausii* (previously classified as *B. subtilis* species, a constituent of the probiotic Enterogermina®) is a mixture of spore forms of strains of *B. subtilis* given orally as a pharmaceutical probiotic.

These microorganisms do not colonize the human intestine but have a transient presence in ingested foods. Spinosa et al. (2000) inoculated intragastrically two groups of mice with a single dose of 10⁹ spores of either *B. clausii* (Enterogermina) or a *B. subtilis* MO1099 derivative. Samples were taken from intestinal sites, lymph organs, and blood at 4, 24, and 72 h after inoculation. Spores of the inoculated *Bacillus* strains were found at all intestinal sites at 4 h. They were largely excreted in the feces as spores, with their presence decreasing exponentially over 72 h, by when they were a 10th of the total spore count. The minimum inhibitory concentration of conjugated bile salt taurodeoxycholic acid for the *Bacillus* strains was 100- to 1000-fold lower than those of normal
human intestinal bacteria, a possible explanation for the lack of spore germination and intestinal growth. Bacteria and spores were not detected at significant levels in blood. In one mouse, *B. clausii* was detected in the mesenteric lymph nodes and spleen with a cumulative count of spores and vegetative cells 10-fold higher than the spore count alone. The observation that spores were unable to germinate in the murine intestinal tract but grew vegetatively after translocation underlines the importance of elucidating those factors favorable for translocation of spore-forming organisms and their subsequent germination external to the GIT, particularly where the genus may have pathogenic members. Blood cultures positive for *Bacillus* in an immunocompromised patient previously treated with Enterogermina have been reported (Oggoni et al. 1998).

Antibiotic susceptibility patterns of potential probiotic *Bacillus* strains have also been determined (Sorokulova et al. 2008; Tompkins et al. 2008). Antibiotic resistance of *B. clausii* to certain antibiotics has been shown to be chromosome encoded and not linked to transferable genetic elements (Girlich et al. 2007). Mechanisms of resistance have been also studied in clostridia for the β-lactamases. Resistance to penicillin is especially common in *Clostridum butyricum*. This microorganism produces β-lactamases that are inducible by some β-lactam antibiotics and inhibited by sulbactam.

Rigorously designed studies are needed to characterize and demonstrate the efficacy and safety of spore-forming probiotic bacteria.

### 21.5 Probiotics in Animals

The use of probiotics in food animals and aquaculture is well established. Probiotics are reported to improve general health, increase growth and weight gain, and suppress pathogens. Overuse of antibiotics in animal husbandry and the possibility of antibiotic resistance have heightened interest in probiotics as alternatives. It has been suggested that the use of probiotics in food animals can reduce the risk of pathogen transfer from food to humans (Atlas 1999). Conversely, transferable antibiotic resistance determinants from strains of animal probiotics potentially harboring these genes may also in theory enter the human food chain (Ouwehand and Salminen 2003).

In the EU, the legislation and recommendations for the safety of probiotics for animals is far stricter than for humans. The European Commission (EC) Scientific Committee on Animal Nutrition (2003) has recommended that, before a bacterial strain can be accepted as an animal probiotic, the intrinsic or acquired genetic basis of observed resistance to representative antibiotics and its transferability should be determined. For species with known intrinsic resistance to an antibiotic, such as vancomycin resistance in lactobacillus, the absence of known resistance genes should be confirmed. Strains carrying acquired resistance to antibiotics used in veterinary or human medicine should not be used in microbial feed additives, except when the basis of resistance is a mutation on a gene intrinsic to that bacterium. This policy would exclude from use any probiotic feed additive containing one or more bacteria with resistance genes capable of being transferred to other bacteria.

Aarestrup et al. (2000) tested *E. faecalis* and *E. faecium* isolated from humans, chickens, and pigs for susceptibility to 12 different microbial agents, and the presence of genes encoding resistance. The same combinations of antibiotic resistance were observed among isolates from human and animal origin and the same genes encoding resistance were detected. The frequent detection of similar resistance patterns and genes indicated that transmission of resistant enterococci or resistance genes occurs between humans, chickens, and pigs.

The fact that probiotic feed additives are used in agriculture and aquaculture does not obviate the need for them to be safe for humans. Fish and animal probiotics have the potential to cross into
the human food chain, or transfer antibiotic resistance, and thus should be demonstrated as safe in animals and humans. In addition, companion animals live in close relationship with their owner, thus a chance of cross contamination exists and the probiotic has to be safe both for the pet and its owner. Rinkinen et al. (2003) evaluated the in vitro ability of LAB strains to inhibit adhesion of canine and zoonotic pathogens to canine small intestinal mucus. Unexpectedly, *E. faecium* M74 and *E. faecium* SF273 both significantly enhanced the percentage adhesion of *Campylobacter jejuni*. Companion animals are thought to be a reservoir of *C. jejuni* and many veterinary probiotics contain *Enterococcus* species. The observation that *E. faecium* enhanced rather than excluded the adhesion of *C. jejuni* suggests that *E. faecium* may be a risk factor in human campylobacter infection.

### 21.6 Probiotics and Infection

It is essential that a probiotic should not have the ability to invade the host cells and cause infection. What is its pathogenic potential? Do other strains or related species cause clinically important infections or produce toxins? Pathogenic microorganisms can be found all around the domain *Bacteria*, indicating the lack of common "pathogenicity" determinants and making the identification of all the potentially pathogenic microorganisms difficult. It is therefore important to identify the pathogenicity traits associated with any specific microorganism. In some studies similar properties have been found between clinical isolates and commercial probiotic strains (Ouwehand et al. 2004a, 2004b), indicating that not only bacterial factors but also factors associated with the host may play a role. It has been recently shown that both microbiota composition and immune status of the host may be involved in determining the safety of specific probiotic strains (Gronbach et al. 2010). In this context, it is necessary to clearly identify the possible risks associated with each probiotic strain, as different strains can possess different characteristics.

This is a significant issue where the intestinal barrier is immature, as in preterm infants; where its integrity is impaired from radiotherapy, antibiotic treatment, or disease; and in immunocompromised states, such as human immunodeficiency virus (HIV) infection. With advances in medical care, an increasing proportion of the community may be immunocompromised at some time, or at risk of opportunistic infection.

Recent results suggest that enteral administration of probiotics to patients with severe acute pancreatitis or patients in high risk of developing bowel ischemia may be especially risky (Besselink et al. 2008). Pancreatitis is a severe, potentially lethal disease that is characterized by bacterial overgrowth and mucosal barrier failure on the small intestine. Some preliminary studies suggested a potential beneficial effect of probiotics in this condition (Olah et al. 2002). However, in a recent large multicenter, randomized, double-blinded clinical trial of patients with severe acute pancreatitis, prophylaxis with a mix of six species doubled the mortality rate in the treatment group in comparison with the placebo group (Besselink et al. 2008). The study cohort included 296 patients with acute pancreatitis with a predicted severe course of disease. Patients were randomly assigned to receive treatment of 10 billion bacteria per day (probiotic, *n* = 152) or placebo (*n* = 144) administered enterally for 28 days. There were 24 deaths in the probiotic group and 9 in the placebo group; more than 80% of deaths were due to multiorgan failure. Bowel ischemia was detected during surgery or autopsy in nine patients in the probiotics group and in none of the patients in the placebo group. The authors speculated that administration of probiotic bacteria along with enteral nutrition might have increased local oxygen demand, with a combined deleterious effect on an already critically reduced blood flow. Another possibility is an inflammatory response to the probiotic bacteria, again with a further reduction of capillary blood flow and ultimately ischemia. The
researchers concluded that probiotics should not be administered routinely in patients with predicted severe acute pancreatitis and that probiotics can no longer be considered harmless adjuncts to enteral nutrition, especially in critically ill patients or patients at risk of nonocclusive mesenteric ischemia. Although these results should be considered with caution and are not applicable to other probiotic applications or modes of administration, this study highlights the need for careful strain-by-strain safety evaluation, as well as a continuous long-term follow-up.

Although rare, some clinical cases have been reported in which strains indistinguishable from ingested probiotic strains, such as *Saccharomyces boulardii* or *Lactobacillus rhamnosus* GG, have been identified in association with infection (Lahtinen et al. 2009).

A valuable adjunct to future epidemiological studies such as that by Salminen et al. (2002), discussed later in Section 21.9 (Epidemiological Surveillance), would be an analysis of what relationship if any, may exist between the clinical status of the patient and the presence of *Lactobacillus* bacteremia. *Lactobacillus* species in general are thought to have low pathogenicity or be opportunistic pathogens in immunocompromised individuals or those with serious underlying disease. It has been suggested that *L. rhamnosus* in particular warrants surveillance because it is associated with more lactobacillemias than other lactobacilli. *L. rhamnosus* is among the most common *Lactobacillus* species in the human intestine, so this may be relative to its extensive presence in the intestine (Salminen et al. 2003).

### 21.7 Antibiotic Resistance

The potential exists for viable probiotics to colonize the intestinal tract and transfer genetic material. Therefore, one of the main targets of the *in vitro* safety assessments of probiotics is the determination of antibiotic resistance properties. Probiotics should be systematically screened for antibiotic resistance susceptibility, since the ability of resistance genes to transfer in the food and gut environment has been demonstrated. The presence of intrinsic antibiotic resistance genes is not a major safety concern in itself, as long as the genes are not mobilized and transferred to other bacteria. However, the current methodologies may not always unequivocally demonstrate the absence of transfer, which can be completely different under *in vitro* and *in vivo* conditions. Thus, it is of great interest to investigate whether probiotics can act as reservoirs for antibiotic resistance genes, from which they could be spread to opportunistic or pathogenic bacteria. The EFSA considers that the nature of any antibiotic resistance determinant present in a candidate microorganism for QPS status evaluation needs to be determined (EFSA 2007). In addition, the antibiotic resistance profiles of probiotic organisms should be known to facilitate a rapid treatment in case it is needed.

In aerobic bacteria conjugation mediated by plasmids or R factors has been documented as the most widespread system for transfer of antibiotic resistance. Antibiotic resistance mechanisms, their genetic nature, and transfer characteristics of resistance determinants have been studied comparatively recently in anaerobic bacteria. It has been shown that the plasmid that encodes for macrolide resistance can be transferred from *L. reuteri* to *E. faecium* and from *E. faecium* to *E. faecalis* in the mouse gastrointestinal tract (Donohue et al. 1998). As previously mentioned, transmissible resistance of enterococci to glycopeptide antibiotics such as vancomycin and teicoplanin is of particular concern, as vancomycin is one of the remaining effective antibiotics for the treatment of multidrug-resistant pathogens (Salminen et al. 2003).

LAB are naturally resistant to many antibiotics by virtue of their structure or physiology. In most cases the resistance is not transferable and the species are also sensitive to antibiotics in clinical use. However, it is not always easy to identify the strains that deserve further study through presentation of atypical resistances. A major step in the differentiation between intrinsic
and acquired antibiotic resistance in probiotic bacteria is the determination and the comparison of antibiotic susceptibility patterns of representative numbers of different strains from each species. Unfortunately there is still a lack of agreement on the resistance susceptibility breakpoints for most antibiotics in probiotic microorganisms. However, major advances in this field have been achieved during recent years to harmonize methods for antimicrobial susceptibility testing in probiotics, and new susceptibility breakpoints for some species of \textit{Lactobacillus} and \textit{Bifidobacterium} have been proposed (Kläre et al. 2007; Mättö et al. 2007).

To summarize, the ability of probiotic strains to transfer antibiotic resistances to pathogenic bacteria must always be taken into account when assessing their safety. Bacterial products must be examined to determine the susceptibility of the strains to relevant antimicrobials. The detection of minimal inhibitory concentrations above the breakpoint requires further investigations to make the distinction between acquired and intrinsic resistance. Nevertheless, the potential for gene transfer is difficult to assess in \textit{vivo}. It is also difficult to assess what level of gene transfer, if any, may be considered acceptable by the community. This is a significant reason to select strains lacking the potential to transfer genetic determinants of antibiotic resistance. There is little basis for scientific regulation of strains with intrinsic resistance, as little is known about the levels of intrinsic resistance in current probiotic and food strains.

21.8 Clinical Studies

Clinical studies in humans have investigated the effect of oral administration of probiotics on the balance of intestinal microflora and in a variety of disorders. Although the principal outcomes in these intervention trials focus on the health benefits of probiotics, the safety of the probiotic administration and the potential adverse events are sometimes reported as a secondary outcome (Rautava et al. 2002; Peng and Hsu 2005; Kajander et al. 2008). Clinical trials focusing on safety enable the \textit{in vivo} evaluation of the effects of probiotics in a controlled manner. Although far from numerous, results of such trials have been published, often carried out with healthy volunteers (Wolf et al. 1995; Mäkeläinen et al. 2003; Burton et al. 2006) and reporting an absence of adverse effect. Lack of tetracycline resistance gene transfer during concomitant ingestion of \textit{L. acidophilus}, \textit{B. animalis}, and antibiotics has also been shown (Saarela et al. 2007).

Longer-term safety studies assessing the effects of probiotic administration on the subsequent growth of children have also been published (Laitinen et al. 2005). Nevertheless, these necessary long-term safety studies are still very rare.

Safety of the probiotics may be of particular interest in specific age groups, such as preterm infants, who have a compromised immune system. In neonates and low-birth-weight infants, successful clinical interventions have been carried out (Hoyos 1999; Agarwal et al. 2003), but serious adverse events have not been reported. Clinical trials suggest that probiotics are also safe to use in follow-up formulas and growing-up milks (Haschke et al. 1998).

Clinical evaluation of probiotics in elderly populations is of special interest since elderly subjects commonly have health-related problems, including infections and gastrointestinal problems, and may also have altered dietary habits and gut microbiota composition compared with healthy adults. For these same reasons, elderly subjects in particular may benefit from the use of probiotics. The safety and the lack of adverse events following the consumption of probiotic strains by elderly subjects has been demonstrated (Gill et al. 2001; Pitkälä et al. 2007).

Several studies carried out with immunocompromised patients have not shown any detrimental effects. Wolf et al. (1998) assessed the safety of \textit{L. reuteri} in HIV adults and found the organism
to be well tolerated with no significant safety problems. In a review of probiotic safety, Borriello et al. (2003) found no published evidence that immunocompromised patients had an increased risk of opportunistic infection from probiotic lactobacilli or bifidobacteria. Until recently many studies were of inadequate design and produced unreliable data. It is important to underline that clinical studies must have an appropriate design and the strains used, as well as the dose, should be clearly indicated. The gold standard is a controlled study with randomized, blind assignment to treatment, placebo, and untreated groups.

21.9 Epidemiological Surveillance

Some studies have investigated the incidence of infections associated with LAB. In the first study, 16S rRNA methods were used to characterize and identify LAB isolated from blood cultures of bacteremic patients in Southern Finland (Saxelin et al. 1996a). The total number of infections caused by lactobacilli was extremely low, and the probiotic strain newly introduced in fermented milks was not associated with infections. In a subsequent study, lactobacilli isolated from bacteremic patients between 1989 and 1994 were compared with common dairy or pharmaceutical strains (Saxelin et al. 1996b). From a total of 5192 blood cultures, 12 were positive for lactobacilli, an incidence of 0.23%. None of the clinical cases could be related to lactobacilli strains used by the dairy industry. In both studies, patients with LAB bacteremia had other severe underlying illnesses.

More recently studies carried out in Scandinavia (Salminen et al. 2002; Sullivan and Nord 2006) examined the incidence of lactobacilli bacteremia in the population for a period corresponding to a rapid increase in consumption of probiotic lactobacilli. In both studies no increase in the incidence or proportion of *Lactobacillus* bacteremia was observed over the study period, and the level of lactobacilli bacteremia remained below 1% of total bacteremia cases. These studies provide evidence that the increased consumption of probiotic lactobacilli had not led to a corresponding increase in *Lactobacillus* bacteremia.

21.10 How Can We Compare Probiotics?

Many bacteria are tested to find a putative probiotic, yielding conflicting data, sometimes for the same organism. Comparisons between studies and organisms cannot be readily made because of nonstandardized dosing procedures, particularly for the number of bacteria and the duration of dosing. Pharmacokinetics, pharmacodynamics, safety, and the risk of acquisition of antimicrobial resistance have usually not been evaluated (Sullivan and Nord 2002).

Probiotic effects are strain specific, illustrating the need to characterize the relationship between the dose, its duration and effect, on a strain-by-strain basis. When considering the pharmacokinetics of the probiotic organism, we want to know if the bacterial strain modifies intestinal microbiota. In determining the dose–response relationship, if there is failure to elicit an effect, is that because the organisms failed to reach effective levels at the site, or is it due to rapid elimination of the bacteria, or nonpersistence, or destruction?

It is unclear whether the proposed consumption of a probiotic is to be done on a regular daily basis for the whole of life, or irregular and dependent on symptoms. Information is not readily available on the equivalence or comparability of formulations in different preparations; on the distinction between spore or vegetative forms, powders, granules, tablets, liquids, and yogurts; or adult and pediatric products. Intake data are not generally available for those countries where
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products are used. Nutritional studies may be needed in addition to toxicological studies, depending on the nature of the product, its intended use, its anticipated intake, the impact of dietary intake on the spectrum of colonic flora, their metabolic functions, and bioavailability of nutrients (International Life Sciences Institute 2001).

21.11 Evolution of Guidelines for Probiotic Safety

Before new probiotic microorganisms and novel probiotic products are introduced into the market, their safety will need to be assured. There is vigorous debate on what constitutes appropriate safety testing for novel probiotic strains proposed for human consumption. Conventional toxicology and safety evaluation is of limited value in assessing the safety of probiotic bacteria.

In 1996 the EU initiated the program Demonstration of Nutritional Functionality of Probiotic foods, PROBDEMO CT96-1028, the aim of which was to provide and verify scientific evidence of claims for probiotic products. It established a list of safety criteria for probiotic foods, as seen in Table 21.1.

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<tr>
<th>Table 21.1 PROBDEMO Criteria for Safety of Probiotics</th>
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<tr>
<td>1. The producer of food has the ultimate responsibility for supplying a safe food. Probiotic foods should be as safe as other foods.</td>
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<td>2. If a probiotic food is a novel food, it is subject to legal approval according to the EU directive for novel foods.</td>
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<td>3. When a strain has a long history of safe use, it will be safe as a probiotic strain and will not result in a novel food.</td>
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<tr>
<td>4. The best test for food safety is a well-documented history of safe human consumption. When a strain belongs to a species for which no pathogenic strains are known and for which other strains have been described that have a long history of safe use, it is likely to be safe as a probiotic food and will not result in a novel food.</td>
</tr>
<tr>
<td>5. When a strain belongs to a species for which no pathogenic strains are known but which do not have a history of safe use, it may be safe as a probiotic food but will result in a novel food and should be treated as such.</td>
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<tr>
<td>6. When a new strain belongs to a species for which strains are known that are pathogenic, it will result in a novel food.</td>
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<tr>
<td>7. State of the art taxonomy is required to describe a probiotic strain, including DNA–DNA hybridization and rRNA sequence determination. This reasoning specifically applies to mutants of a probiotic strain.</td>
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<tr>
<td>8. In line with recommendation (1), strains that carry transferable antibiotic resistance genes (genes encoding proteins that inactivate antibiotics) should not be marketed.</td>
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<tr>
<td>9. Strains that have not been properly taxonomically described using the approaches indicated in (7) should not be marketed.</td>
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<td>10. Strains should be deposited in an internationally recognized culture collection.</td>
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The EC’s 5th Framework Program addressed this complex subject in its collaborative project “Biosafety evaluation of probiotic LAB used for human consumption” of the PROEUHEALTH cluster, where the presence and horizontal transfer of antibiotic resistance genes, presence and detection of virulence factors, evaluation of adverse immune effects and probiotic survival, colonization, and genetic stability in the human gut were evaluated.

The FAO and WHO convened a joint FAO-WHO Working Group to draft guidelines for evaluating probiotics used in food (FAO-WHO 2006). The working group proposed a framework of strain identification and functional characterization, followed by safety assessment and phase 1, 2, and 3 human trials. It recommended that probiotic foods be properly labeled with the strain designation, minimum numbers of viable bacteria at the end of shelf life, storage conditions, and manufacturer’s contact details. The minimum tests required for characterization of safety are set out in Table 21.2. The working group further considered that assessment of lack of infectivity by a probiotic strain in immunocompromised animals would increase confidence in the safety of the probiotic.

The EC developed a scheme, with some similarity to the GRAS system in the United States, to formulate a consistent approval procedure for the use of microorganisms in feeds and foods (EFSA 2007). Microorganisms associated with animal feeds are strictly regulated in Europe, but there was no formal mechanism for granting safety status to microorganisms in human food. This led to inconsistencies where an organism with a long history of safe use in human foods was subjected to strict safety assessment as an animal feed additive.

The scheme proposed is based on the concept of QPS, defined as “an assumption based on reasonable evidence” and qualified to allow certain restrictions to apply. This approach establishes a consistent generic safety assessment of microorganisms without compromising safety standards. Case-by-case evaluations would be limited to aspects particular to the organism, obvious examples being acquired antibiotic resistance determinants in LAB or toxin production in species known to contain toxigenic strains.

Broadly, the characteristics to be evaluated for QPS approval are

- Unambiguous identification at the claimed taxonomic level
- Relationship of taxonomic identity to existing or historic nomenclature

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<tr>
<th>Table 21.2 Joint FAO-WHO Working Group Recommendations to Evaluate Probiotic Safety</th>
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<tr>
<td>1. Determination of antibiotic resistance patterns.</td>
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<tr>
<td>2. Assessment of metabolic activities (e.g., ( \delta )-lactate production, bile salt deconjugation).</td>
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<tr>
<td>3. Assessment of side effects during human studies.</td>
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<tr>
<td>4. Post-market epidemiological surveillance of adverse incidents in consumers.</td>
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<tr>
<td>5. If the strain under evaluation belongs to a species that is a known mammalian toxin producer, it must be tested for toxin production.</td>
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<tr>
<td>6. If the strain under evaluation belongs to a species with a known hemolytic potential, determination of hemolytic activity is required.</td>
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</table>

Some Considerations for the Safety of Novel Probiotic Bacteria

- Degree of familiarity with organism, based on weight of evidence
- Potential for pathogenicity to humans and animals
- End use of the microorganism

QPS status would not apply to a microorganism that commonly causes pathogenicity. If pathogenicity was limited to selected strains, and its mechanism was testable, the microorganism might remain eligible for QPS status with qualifications.

An example of how the process could proceed is summarized for *B. subtilis*. Sufficient information is available to establish the identity of strains in the *B. subtilis* group. The biology and pathogenicity of this group are understood well enough to exclude problem strains. Some strains may qualify for QPS status, provisional to meeting the qualifications that PCR-based evidence shows an absence of toxigenic potential; production strains with toxigenic potential fail to produce detectable toxin levels in the production system employed; the strain is free of acquired resistance to antibiotics of significance to human and veterinary medicine; and it has an absence of capacity to produce antibiotics with structural similarities to those in human or veterinary medicine likely to encourage development of resistance.

### 21.12 Safety Considerations in Summary

- Probiotic bacteria to be unequivocally identified and defined with correct taxonomy.
- Probiotic strains to be deposited in a recognized international culture collection for access by manufacturers, scientists, and regulators to ensure organisms can be monitored for genetic drift and comparison with clinical isolates.
- Novel probiotic strains from species with pathogenic, toxigenic, or other adverse properties to be evaluated with scientific rigor.
- Probiotic organisms to be systematically screened for antibiotic resistance and its transference.
- Immunomodulatory effects of probiotics to be assessed in defined target populations.
- Clinical studies should comply with the gold standard of randomized, double-blind placebo controlled design.
- Probiotics in animal feed additives or veterinary products should be evaluated for their safety in the human food chain.
- Labeling of probiotic products should accurately reflect content, shelf life, claimed attributes, and dose.
- Following the introduction of novel probiotics, intake data should be gathered, especially for long-term consumption.
- After market release of a novel probiotic, epidemiological surveillance for any associated adverse effects, particularly infection, should be instituted.
- Characterization of clinical isolates for comparison with endogenous and probiotic strains as integral to confirming its safety.
- National reference centers to identify species and strain in clinical cases.
- National clinical and epidemiological databases to include identity of organism, status of patient’s underlying conditions, coexisting infections, and outcomes.
References


EFSA. 2007. Opinion of the Scientific Committee on a Request from EFSA on the introduction of a Qualified Presumption of Safety (QPS) approach for assessment of selected microorganisms referred to EFSA. EFSA J 587:1–16.


Some Considerations for the Safety of Novel Probiotic Bacteria


Chapter 22

Probiotics and Human Immune Function

Harsharnjit S. Gill, Jaya Prasad, and Osaana Donkor

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22.1 Introduction

The human gastrointestinal tract (GIT) represents the largest body surface area exposed to the external environment. It is continuously challenged by a diverse array of environmental, dietary, and microbial antigens (derived from the enteric flora). In addition to facilitating digestion and allowing absorption of nutrients, the GIT performs a very complex and delicate function of exhibiting tolerance to innocuous environmental antigens and enteric flora, but at the same time mounting an aggressive immune response toward pathogenic organisms. Failure to tightly regulate these responses leads to enhanced susceptibility to infectious diseases and immunoinflammatory disorders. To perform these functions efficiently, the GIT is endowed with the largest immune system in the body.

Immediately after birth, the immune system of the newborn is immature and functionally naive. Signals provided by the commensal flora and environmental antigens play a central role in the postnatal development of the gut immune system and establishment of immunoregulatory pathways. Defective immunoregulation resulting from reduced or aberrant exposure to microbes during early life is associated with an increased incidence of atopic and autoimmune disorders (Rook et al. 2005). The role of gut microflora in immune regulation is also highlighted by distinct patterns of gut microflora in infants with enhanced propensity for developing atopic diseases later in life compared with healthy infants (Kalliomäki et al. 2001). Little is known about the bacterial species or bacterial molecules that are critical for the postnatal development and/or activation of the mucosal immune system. Evidence from recent studies suggests that this function might be associated with the presence of limited types of intestinal bacteria. For example, colonization with just *Bacteroides fragilis*, a ubiquitous gut microorganism, was found to be effective in directing lymphoid organogenesis and correcting systemic T-cell deficiencies and Th1/Th2 imbalances in germ-free mice (Mazmanian et al. 2005).

Emerging evidence shows that inflammation and alterations in immune capacity are the root cause of most infectious, inflammatory, and autoimmune disorders, including metabolic diseases. Thus, there is increasing interest in developing intervention strategies that are effective in regulating dysregulated or overexpressed immune responses (polarized Th1, Th2, Th17 responses), and enhancing immune competence in individuals with less than adequate immune function. Studies over the past 20 years have shown that specific strains of probiotics are endowed with specific immunomodulatory properties and could be used to optimize immune function in health and disease. Probiotics are defined as live microorganisms that, when administered in adequate amounts, confer a health benefit on the host (Food and Agriculture Organization/World Health Organization [FAO/WHO] 2001). The bacterial strains most commonly used as probiotics are *Lactobacillus* and *Bifidobacterium*. The primary objective of this chapter is to provide an overview of the effect of probiotics on human immune function in health and disease and discuss mechanisms by which these effects are mediated.

22.2 Probiotics and Modulation of Immune Function

The effects of probiotics intake on immune function have been the subject of numerous investigations in healthy subjects and subjects with immunoinflammatory disorders. The results of these studies have been quite heterogeneous due to the use of different experimental protocols/designs, probiotics strains, dose and duration of treatment, study subjects, immune parameters and disease end points measured, and host factors (such as immune status, host genetics, lifestyle, etc.). However, there is unequivocal evidence to suggest that specific strains of probiotics are able to
influence a wide array of immune functions in health and disease and could be effectively used for optimizing human health.

### 22.2.1 Effect on Mucosal Immunity

#### 22.2.1.1 Intestinal Epithelial Cells

A single layer of intestinal epithelial cells (IECs) separates the host from the external environment. In addition to performing a barrier function, IECs play an important role in regulating innate and acquired immune responses in the GIT. They possess the capacity to recognize and respond to different bacteria and bacterial products in a discriminatory manner.

Thus, the activation status and regulatory function of IECs is dependent on the nature of bacterial stimuli received. Several *in vitro* studies have shown that probiotics stimulation of IECs leads to the release of a variety of pro- and/or anti-inflammatory cytokines depending on the probiotics strain used. For example, interleukin (IL)-8 was the major cytokine produced by enterocytes (HT-29 and Caco-2) following exposure to *Lactobacillus* GG (Zhang et al. 2005), *L. sakei* (Haller et al. 2000), and *L. plantarum* 299v (McCracken et al. 2002). In contrast, *L. reuteri* inhibited IL-8 production in HT-29 cells (Ma et al. 2004). VSL#3-derived DNA, a probiotics mixture with proven efficacy against pouchitis and ulcerative colitis (UC), has also been shown to reduce expression of IL-8 in IECs; the effect was mediated through inhibition of nuclear factor \( \kappa B \) (NF\( \kappa B \)) activation (Jijon et al. 2004). Induction of immune tolerance toward ingested *L. plantarum* in humans has also been shown to involve the modulation of NF\( \kappa B \)-dependent pathways in the duodenal mucosa (van Baarlen et al. 2009). Together, these observations suggest that, by inducing the release of various cytokines and chemokines by IECs, probiotics are able to influence the functioning of the mucosal and systemic immune responses.

#### 22.2.1.2 Gut Mucosal Barrier Function

A stable gut mucosal barrier is essential for inhibiting aberrant transfer of dietary and microbial antigens across the gut mucosa and preventing activation of inappropriate, host-damaging immune responses. Impaired gut barrier function is a characteristic feature of food allergies and immunoinflammatory gut diseases (Graschwitz and Hogan 2009).

Several studies have shown that ingestion of specific probiotics is effective in preventing and repairing mucosal damage caused by food allergens (Rosenfeldt et al. 2003), nonsteroidal anti-inflammatory drugs (Montalto et al. 2004) and following hemorrhagic shock (Luyer et al. 2005). Probiotics have been shown to mediate these effects by stimulating production of secretory IgA (sIgA) (Malin et al. 1996; Vijanen et al. 2005) and other host defense molecules such as defensins (Schlee et al. 2008), attenuating pro-inflammatory responses (Pena et al. 2005; Sheil et al. 2004; Liu et al. 2010), inducing production of mucin (Mack et al. 2003) and cytoprotective proteins (Yan et al. 2007), protecting integrity of tight junctions (Montalto et al. 2004; Luyer et al. 2005), promoting tissue repair (Yamaguchi et al. 2003; Otte and Podolsky 2004), reducing bacterial adhesion and cytoskeletal rearrangements (Sherman et al. 2005), and by secreting trophic factors and nutrients.

#### 22.2.1.3 IgA Production

sIgA is the main immunoglobulin found at mucosal surfaces and its levels in saliva are widely used as an indicator of mucosal immunity (Albers et al. 2005). A relationship between the level of fecal
slgA antibody and enhanced virus-neutralizing capacity and increased viral clearance (Colomina et al. 1998) has also been reported. Thus, a lack of nonspecific slgA at the mucosal surface or the inability to produce specific slgA can lead to an increased risk of infection and intestinal inflammatory disorders.

Many probiotic bacteria have been tested for their ability to augment mucosal immune responses in human subjects at different life stages ranging from infancy to old age. During a variable period after birth, the mucosal immune system is functionally immature (Cummings et al. 2004). For example, newborns exhibit reduced capacity to generate IgA-producing cells. The numbers of IgA-producing cells increases progressively in response to intestinal antigenic stimulation particularly when oral food intake begins and the gut microbiota establishes (Isolauri 2007). Fukushima et al. (1998) reported that children (aged 15–31 months old) fed follow-up formula containing Bifidobacterium showed higher levels of total IgA in feces during intervention as compared with preintervention levels. Similar results were reported by Lara-Villoslada et al. (2007) in 3–12-year-old children fed L. coryniformis CECT5711 and L. gasseri CECT5714. In contrast, Bakker-Zierikzee et al. (2006) found no significant effect of infant formula supplemented with B. animalis Bb12 on fecal slgA in 0–32-week-old infants. Whether this was due to the strain and dosage of probiotics used or some other reason is not clear.

The effect of probiotics on mucosal immunity in healthy adults and the elderly has also been the focus of many studies (Table 22.1). Recently, Kotani et al. (2010) in a randomized, double-blind placebo controlled dietary intervention trial involving elderly subjects showed that ingestion of sterile water beverage containing heat-killed probiotic L. pentosus b240 was effective at significantly improving the levels of average salivary slgA secretion rate (p = .024) as well as slgA concentration (p = .01). Ouwehand et al. (2009), using L. acidophilus NCFM and lactitol dietary intervention in elderly subjects, reported similar effects. In another study feeding of B. lactis HN019 and L. rhamnosus HN001 to pregnant mothers recruited 2–5 weeks before delivery and continuing for 6 months in lactation was found to significantly enhance IgA levels (Prescott et al. 2008). Contrary to these observations, however, several studies using different strains (L. johnsonii La1 [Marteau et al. 1997]; B. animalis ssp. lactis Bb12 and L. paracasei ssp. paracasei CRL431 [Christensen et al. 2006]; L. gasseri CECT 5714 and L. coryniformis CECT 5711 [Olivares et al. 2006b]) have reported no effects of probiotic supplementation on the mucosal immunoglobulin levels in healthy adults. These differential effects in children and the elderly versus healthy adults reinforces the view that the probiotic effects are more pronounced in the population groups with less than optimal immune function. slgA plays a key role in host protection at mucosal surfaces and maintaining mucosal homeostasis.

### 22.2.2 Effect on Systemic Immunity

#### 22.2.2.1 Phagocytic Activity

The ability of probiotics to enhance phagocytic activity of peripheral blood leukocytes (monocytes/macrophages and polymorphonuclear [PMN] cells) in healthy subjects, especially with less than adequate immune competence, has been demonstrated in a number of human studies (Table 22.1).

Intake of L. johnsonii La1, L. acidophilus 74-2, B. lactis 420, B. lactis Bb12, L. rhamnosus HN001, or B. lactis HN019 resulted in enhanced phagocytic capacity of peripheral blood leukocytes (PMN and monocytes) in healthy subjects (Schiffrin et al. 1995; Donnet-Hughes et al. 1999;
Table 22.1 Immune-Enhancing Effects of Probiotics

<table>
<thead>
<tr>
<th>Reference</th>
<th>Intervention</th>
<th>Study Population</th>
<th>Study Design</th>
<th>Immune Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amati et al. (2010)</td>
<td>Fermented milk containing <em>Lactobacillus rhamnosus</em> GG and oligofructose for 1 month</td>
<td>10 free-living elderly subjects</td>
<td>Before and post-intervention</td>
<td>↑ IL-1, IL-6, and IL-8</td>
</tr>
<tr>
<td>Sierra et al. (2010)</td>
<td>Dose of $2 \times 10^8$ CFU of <em>L. salivarius</em> CECT5713 in capsules during 4 weeks</td>
<td>40 healthy adults</td>
<td>Randomized, double blind, placebo controlled</td>
<td>• ↑ %NK cells and monocytes</td>
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<td>• ↑ Serum immunoglobulin M, A, and G</td>
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<td>• ↑ IL-10</td>
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<tr>
<td>Kotani et al. (2010)</td>
<td>Sterile water beverage containing heat-killed <em>L. pentosus</em> b240 (4 × $10^9$ cells) daily for 12 weeks</td>
<td>80 healthy adults with mean age about 70 years</td>
<td>Randomized, double blind, placebo controlled. Divided into two groups. Treatment or b240 group $n = 40$ and placebo group $n = 40$.</td>
<td>Significantly ($p &lt; .001$) greater changes in sIgA in the b240 group than in the placebo group over the intervention period.</td>
</tr>
<tr>
<td>Ibrahim et al. (2010)</td>
<td>Commercial probiotic cheese containing approximately $10^9$ CFU/day of <em>L. rhamnosus</em> HN001 and <em>L. acidophilus</em> NCFM for 4 weeks</td>
<td>31 (21 female and 10 male) healthy elderly volunteers aged 72–103 (median 86)</td>
<td>Three phases. In phase 1, the subjects consumed control cheese during breakfast for 2 weeks (run-in). In phase 2, the subjects consumed a probiotic cheese for 4 weeks (intervention). In phase 3, the subjects consumed the same control cheese again for 4 weeks (washout).</td>
<td>• ↑ Cytotoxicity of NK cells</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>• ↑ Phagocytosis</td>
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<tr>
<th>Reference</th>
<th>Intervention</th>
<th>Study Population</th>
<th>Study Design</th>
<th>Immune Effect</th>
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</table>
| Makino et al. (2010) | Yogurt (90 g) fermented with *L. bulgaricus* OLL1073R-1 or 100 ml milk daily over 8 or 12 weeks period | 57 (median age 74.5) and 85 (median age 67.7) healthy elderly individuals in two separate studies | Randomized, double blind, placebo controlled                                    | ↑ NK cell activity in yogurt group 
(p = .028)                                                                                              |
| Ouwehand et al. (2009) | A combination of lactitol and *L. acidophilus* NCFM for twice daily for 2 weeks | Healthy free-living elderly subjects. Placebo n = 23 mean age 71.7; symbiotic n = 24; mean age 70.3 | Randomized, double blind, and placebo controlled (2 weeks run-in, 2 weeks intervention and 2 weeks washout period) | • Fecal concentration of IgA were found to change significantly (p = .024) over time after correction for baseline differences.  
• Changes in PGE2 levels were also significant (p = .028). |
| Schiffrin et al. (2009) | Probiotic yogurt (2 × 150 g) containing *L. johnsonii* La1 (daily dose of 10^9 CFU) for 4 weeks | 23 participants with positive and 13 participants with negative hydrogen breath test | Before and post-intervention (2-week run-in period followed by 4-week intervention period) | • ↓ In plasma endotoxin and basal phagocytic activity of leukocytes after yogurt intake in both groups.  
• ↑ Cytokine response and free radical response in ex vivo exposed monocytes and neutrophils, respectively.  
• Plasma levels of LPS binding protein and soluble CD14 LPS pattern recognition receptors. |
| Klein et al. (2008)   | Probiotic yogurt (300 g/day) supplemented with *L. acidophilus* 74-2 and *B. lactis* 420 or placebo product for 5 weeks | 26 healthy volunteers (mean age 25 years)                                       | Randomized, double-blind, placebo-controlled, cross-over design               | • ↑ % Phagocytic activity (from 92% to 95%) in granulocytes and monocytes; no change in their oxidative burst activity.  
• No change in specific immune parameters (expression of CD3, CD19, CD4, CD8, CD16, and CD56, CD57, CD3+HLA-DR, CD25, CD122, or CD54). |
<table>
<thead>
<tr>
<th>Study</th>
<th>Intervention</th>
<th>Subjects</th>
<th>Design</th>
<th>Key Findings</th>
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</table>
| Ouwehand et al. (2008)        | Oat-based drink supplemented with $10^9$ CFU/day of both *B. longum* 2C (DSM 14579) and 46 (DSM 14583) | 209 elderly subjects (average age 84.3 years) | Randomized, double-blind, placebo-controlled trial with 3 groups, intervention group $n = 56$; placebo group $n = 67$; control group ($n = 86$). Control group consumed oat-based drink supplemented with *B. animalis* ssp. *lactis* Bb12. | - Negative correlation was observed between level of *Bifidobacterium* species and pro-inflammatory cytokine TNF-α and the regulatory cytokine IL-10.  
- The presence of fecal *B. longum* and *B. animalis* correlated with reduced serum IL-10.  
- The anti-inflammatory TGF-β1 levels were increased over time in all three groups.  
- The presence of *B. breve* correlated with higher serum TGF-β1 levels. |
| Prescott et al. (2008)        | Daily supplements of either *B. lactis* HN019 ($6 \times 10^9$/day) or *L. rhamnosus* HN001 ($9 \times 10^9$/day) | 71 women received probiotic treatment beginning 2–5 weeks before delivery and continuing for 6 months in lactating women. | Randomized, double-blind, placebo-controlled trial (sampling done at 3–7 days, 3 months, and 6 months postpartum). *B. lactis* group, $n = 35$ and *L. rhamnosus* group, $n = 34$ or placebo, $n = 36$. | - Neonates of mothers who received probiotic had ↑CB IFN-γ levels ($p = .026$) compared to placebo group.  
- ↑ Proportion had detectable levels of blood IFN-γ ($p = .03$) compared with placebo group.  
- ↑TGF-β levels in early breast milk in mothers receiving HN019 ($p = .041$) and HN001 ($p = .075$).  
- ↑Levels of IgA in breast milk of mothers receiving HN019 ($p = .008$) and HN001 ($p = .011$).  
- Neonatal plasma sCD14 levels were ↓in HN019 group compared to placebo ($p = .041$). |

(continued)
### Table 22.1 Immune-Enhancing Effects of Probiotics (Continued)

<table>
<thead>
<tr>
<th>Reference</th>
<th>Intervention</th>
<th>Study Population</th>
<th>Study Design</th>
<th>Immune Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ortiz-Andrellucchi et al. (2008)</td>
<td>Milk fermented with <em>L. casei</em> DN114001 for 6 weeks</td>
<td>104 women (18–40 years) recently delivered and breast feeding (<em>n</em> = 59, treatment; <em>n</em> = 45, placebo)</td>
<td>Randomized, double blind, placebo controlled</td>
<td>• ↑ T and B lymphocytes and significant ↑ in NK cell activity</td>
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<td></td>
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<td>• ↓ TNF-α in maternal milk</td>
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<tr>
<td>Kekkonen et al. (2008)</td>
<td>Milk-based drink containing either <em>L. rhamnosus</em> GG (1.6 × 10⁸ CFU/day), <em>B. animalis</em> ssp. <em>lactis</em> Bb12 (3.5 × 10¹⁰ CFU/day) or <em>Propionibacterium freudenreichii</em> ssp. shermanii JS (3.3 × 10¹⁰ CFU/day) or placebo drink for 3 weeks</td>
<td>62 healthy adult volunteers (mean age = 44 years with age range of 23–58). <em>n</em> = 13 in LGG group, <em>n</em> = 17 in JS group; <em>n</em> = 16 in Bb12 group; and <em>n</em> = 16 in control group</td>
<td>Randomized, double blind, placebo controlled</td>
<td>• Serum highly sensitive CRP levels in LGG group compared with Bb12 group at 3 weeks.</td>
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<td>• ↓ Serum highly sensitive CRP levels ↓ in the LGG and JS groups compared with controls (significance not given).</td>
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<td>• No effect on change in serum TNF-α, IL-6, IL-10, or IFN-γ concentrations during the study.</td>
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<td></td>
<td>• No effect on leukocyte, monocyte, neutrophil, basophil, eosinophil, or lymphocyte counts.</td>
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<td></td>
<td>• No effect on serum IgM, IgG, or IgA levels.</td>
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<td>• No effect on sIgA levels in saliva.</td>
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<td></td>
<td>• No effect on IFN-γ, IL-1β, IL-6, IL-8, IL-10, or IL-12 production by <em>Streptococcus pyogenes</em>, influenza A H3N2, or LPS stimulated peripheral blood mononuclear cells (PBMC).</td>
</tr>
</tbody>
</table>
Probiotics and Human Immune Function

- ↓ TNF-α production by *S. pyogenes* stimulated PBMC in the LGG group compared with controls. No effect on production upon stimulation with influenza A.
- H2N3 or LPS, or in other probiotic groups.
- ↓ IL-2 production by influenza A H2N3–stimulated PBMC in Bb12 group compared with all other groups, but no effect on production upon stimulation with *S. pyogenes* or LPS, or in other probiotic groups.

<table>
<thead>
<tr>
<th>Marschan et al. (2008a,b)</th>
<th><em>L. rhamnosus</em> GG (10 × 10⁹ CFU/day), <em>L. rhamnosus</em> LC705 (10 × 10⁹ CFU/day) <em>B. breve</em> Bb99 (4 × 10⁹ CFU/day), or <em>Propionibacterium freudenreichii ssp. shermanii</em> JS (4 × 10⁹ CFU/day), for 2–4 weeks before delivery (mothers). The infants received half of these doses for the first 6 months after birth, plus 0.8 g galacto-oligosaccharides.</th>
<th>Newborn infants with family history of allergy (parental); <em>n</em> = 52 in test group; <em>n</em> = 46 in control group</th>
<th>Randomized, double blind, placebo controlled</th>
<th></th>
</tr>
</thead>
</table>
|  |  |  |  | ✷ Plasma C-reactive protein levels at 6 months (↓ risk of eczema and allergic disease)  
☒ Plasma IL-10 concentration at 6 months  
☒ Total plasma IgA at 6 months  
 appropriates 1-2, IL-4, IL-6, IFN-γ, and TNF-α values were below the detection limit  
☒ No effect on IL-12 or IL-4R expression in cord blood mononuclear cells  
☒ No effect on IL-2, IL-4, IL-5, IL-10, IL-13 or IFN-γ secretion by CB mononuclear cells |

(continued)
<table>
<thead>
<tr>
<th>Reference</th>
<th>Intervention</th>
<th>Study Population</th>
<th>Study Design</th>
<th>Immune Effect</th>
</tr>
</thead>
</table>
| Fukushima et al. (2007)  | *L. johnsonii* La1 NCC533 (10^9 CFU/day for 12 weeks) and *S. thermophilus* (10^8 CFU/day) | Enterally fed elderly patients with dysphasia and dementia; age 75–96 years. Test group *n* = 12 with mean age = 84.4 years. Control group *n* = 12 with mean age = 84.8 years. | Randomized groups                                                                                       | • ↓ Serum TNF-α in test group, but no difference between the groups  
• ↑ Blood phagocytic activity in subjects with initially low levels in the test group after 4 weeks, but no difference between the groups  
• ↑ Erythrocyte numbers at 4 weeks  
• ↑ White blood cell numbers at 12 weeks in the test group, but no difference between groups                                                                 |
| Lara-Villoslada et al. (2007) | Conventional yogurt containing *L. bulgaricus* and *S. thermophilus* or probiotic product (Max Defensas, Puleva Food S. L.) containing *L. coryniformis* CECT5711 and *L. gasseri* CECT5714 for 3 weeks | 30 healthy children (3–12 years old). Fed conventional yogurt for 3 weeks followed by 3 weeks probiotic product.                                                                                             | Before and post-intervention study. Analysis was conducted at baseline (week 0), during yogurt intervention (up to 3 weeks) and then probiotic intervention (4–6 weeks) periods | The inhibition of *S. cholerasuis* adhesion to intestinal mucins was significantly higher (*p* < .05) for fecal waters from children in week 6 compared with samples form weeks 0 and 3. Probiotic consumption was also shown to increase IgA concentration in feces and saliva (*p* < .05). |
| Takeda and Okumura (2007) | *L. casei* Shirota (Yakult; 4 ×10^{10} bacteria/day for 3 weeks)               | Healthy adults with relatively low levels of NK cell activity. Middle aged (*n* = 9; 30–45 years old) and elderly (*n* = 10; 55–75 years old). Divided into treatment and control groups. | Randomized, placebo-controlled, cross-over design                                                      | • In the middle-aged subjects:  
• ↑ NK cell activity (*p* < .01) compared with control group. This was especially true for those who had a low level of NK activity at the start of the study. |
<table>
<thead>
<tr>
<th>Study</th>
<th>Probiotic Strain</th>
<th>Participants</th>
<th>Study Design</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Christensen et al. (2006)</td>
<td><em>B. animalis</em> ssp. <em>lactis</em> (Bb12) and <em>L. paracasei</em> ssp. <em>paracasei</em> CRL-431.</td>
<td>Healthy adults; mean age = 25.6 years with a range of 18–40 year (n = 14 in control group; n = 14 in 10⁸ group; n = 15 in 10⁹ group; n = 13 in 10¹⁰ group; n = 15 in 10¹¹ group)</td>
<td>Randomized, placebo-controlled, parallel, dose response study</td>
<td>No significant effect was observed in immune parameters (e.g., fecal IgA, blood IgA, IgG, or IgM; production of IFN-γ or IL-10 from LPS and/or PHA stimulated blood samples) in any treatment group.</td>
</tr>
</tbody>
</table>
| Clancy et al. (2006) | *L. acidophilus* LAFTI® L10 (2 × 10¹⁰ CFU/day for 4 weeks) | Test group of fatigued athletes (median age = 24.7 years; n = 9). Control group of healthy athletes (median age = 26.1 years; n = 18). Both groups received probiotic supplement. | Randomized, parallel groups | • Secretion of IFN-γ from whole blood cultures was ↑ in the group as a whole. This was ↑ (p = .01) in the group of fatigued athletes compared to levels in the healthy controls.  
• Salivary IFN-γ concentration was non-significantly ↑ in the group as a whole. This was ↑ (p = .03) in the group of healthy athletes, but not in the group of fatigued athletes. |

(continued)
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<tr>
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<th>Study Design</th>
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</table>
| Kim et al. (2006)    | *Bacillus polyfermenticus* (3.1 × 10^8 CFU/day for 8 weeks)                  | Male adult volunteers age range 20–35 years; *n* = 13 in test group, mean age = 25.57 years; *n* = 12 in control group, mean age = 25.21 | Randomized groups | • ↑ Serum IgG concentration  
• ↑ % of CD4+ (Th cells), CD8+ (cytotoxic T cells), and CD56+ (NK cells) in blood  
• No effect on serum IgA or IgM levels  
• No effect on % of T and B cells in blood |
| Meyer et al. (2006)  | Yogurt containing *L. delbrueckii* subsp. *bulgaricus* (6.4 × 10^7 CFU/ml, live) and *S. thermophilus* (3.9 × 10^7 CFU/ml, live), 100 g/day for 2 weeks, then 200 g/day for 2 weeks. Probiotic yogurt containing *L. delbrueckii* subsp. *bulgaricus* (2 × 10^8 CFU/ml), *S. thermophilus* (10^7 CFU/ml) and *L. casei* DN114001 (3.7 × 10^8 CFU/ml), 100 g/day for 2 weeks, then 200 g/day for 2 weeks. | Healthy women, mean age = 24.4 years (range 22–29); *n* = 33; *n* = 17 in probiotic yogurt group; *n* = 16 in conventional yogurt group | Randomized groups | Consumed 100 g/day of either probiotic or conventional commercially available yogurt for 2 weeks and 200 g/day for another 2 weeks followed by a 2-week washout period.  
• ↑ Cytotoxic T cells (CD3+CD16+CD56+) in probiotic group  
• ↑ Natural cytotoxicity of isolated PBMC against K562 leukemic cells in both conventional yogurt and probiotic yogurt groups  
• ↑ IFN-γ production in the probiotic group, from whole blood cultures stimulated with LPS and PHA after the low yogurt intake, but not in the conventional yogurt group  
• ↑ IL-1β production whole blood cultures stimulated with LPS in the conventional yogurt group after the high yogurt intake, but not in the probiotic group  
• ↑ TNF-α production from whole blood cultures stimulated with LPS after low (conventional group) and again after high (both groups) yogurt intakes, and still after 2 weeks of washout (probiotic group) |
IL-10 production by whole blood cultures stimulated with LPS and PHA in the probiotic yogurt group after the low dose period, but ↑ after the washout period

No significant effect on:

- Numbers of total leukocytes (CD45^+), granulocytes, monocytes (CD45^+/CD14^+), total lymphocytes (CD45^+), T cells (CD3^+), Th cells (CD4^+), T reg cells (CD8^+), CD4:CD8 ratio, activated T cells (HLA-DR^+), NK cells (CD16CD56^+)
- Activated T cells (CD69^+ both CD4^+ and CD8^+) in the probiotic group
- IL-6 production by whole blood cultures stimulated with LPS and PHA in either group
- Differences between the groups in production of IFN-γ, IL-1β, TNF-α, or IL-10 by whole blood cultures

Takeda et al. (2006)

Fermented drink (80 ml) containing 4 × 10^{10} CFU of live L. casei Shirota (LcS) for 3 weeks

10 healthy adults aged 69–97 years. Two groups (n = 5) LcS group or placebo group

Randomized, double blind, placebo controlled and crossover trial. Three weeks intervention followed by 7 weeks washout period followed by second intervention.

The ability of LcS to enhance NK cell activity and induce IL-12 production was correlated and the addition of anti-IL-12 monoclonal antibody reduced the enhancement of NK cell activity triggered by LcS.
Table 22.1 Immune-Enhancing Effects of Probiotics (Continued)

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<tr>
<th>Reference</th>
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</thead>
</table>
| Olivares et al.    | • *L. gasseri* CECT 5714 and *L. coryniformis* CECT 5711 (2 × 10^9 CFU of each) plus *S. thermophilus* (10^8 CFU), for 4 weeks | Healthy adults; age 23–43 years; n = 30; included a control group | Randomized, double blind, placebo controlled | • ↑ % of monocytes (CD14^+) after 2 weeks, but not 4 weeks in test group  
• ↑ % of neutrophils in both groups at 2 weeks and in test group at 4 weeks  
• ↑ % of NK cells (CD56^+) in test group at 2 and 4 weeks  
• ↓ % of lymphocytes in both groups at 2 weeks and in test group at 4 weeks, ↓ % of T cells (CD3^+) in test group at 2 and 4 weeks, ↓ % of T memory cells (CD3^+CD45RO^+) in test group at 4 weeks  
• ↑ % of monocytes and granulocytes showing phagocytic activity at weeks 2 and 4 in both groups  
• ↑ Serum IL-10 and IL-4 levels at week 2 in the test group  
• ↑ Serum IgA levels at week 4 in test group  
• ↓ Serum IgE levels at week 2 in both groups  
• No effect on:  
  • Fecal IgA  
  • % of Th cells (CD4^+), Tc cells (CD8^+) or T suppressor cells  
  • (CD4^+CD25^+)  
  • Serum TNF-α or IL-12 levels |
<table>
<thead>
<tr>
<th>Study</th>
<th>Probiotic Strain</th>
<th>Participants</th>
<th>Study Design</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bakker-Zierikzee et al. (2006)</td>
<td><em>B. animalis</em> Bb12 (6 × 10⁹ CFU/100 ml reconstituted formula)</td>
<td>Newborn infants divided into 4 groups. Group 1 (standard formula, n = 19); group 2 (standard formula supplemented with GOS/FOS, n = 19); group 3 (standard formula with probiotic); group 4 (breast-fed infants).</td>
<td>Randomized, double-blind, placebo-controlled study spanning 32 weeks</td>
<td>Infants fed on the probiotic formula showed a highly variable fecal IgA concentration with no statistically significant difference compared with the standard formula group.</td>
</tr>
</tbody>
</table>
| Tylor et al. (2006) | *L. acidophilus* LAVRI-A1 or placebo (maltodextrin) for 6 months | The infants (prone to allergy) received either probiotic (n = 58) or placebo (n = 60) | Randomized, double-blind, placebo-controlled study | - No difference in cytokine response after stimulation with Pansorbin (TLR2) or LPS (TLR4).  
- The mean fluorescence intensities of human leukocyte antigen-DR (HLA-DR) on monocytes, B cells, and dendritic cells (DC) subsets were not affected. |
| de Vrese et al. (2005) | *L. gasseri* PA16/8, *B. longum* SP 073, *B. bifidum* MF 20/5 (Tribion Harmonis™ 5 × 10⁷ CFU/day for at least 3 months during the winter and spring seasons) | Healthy adults not vaccinated with influenza; aged 18–67 years; test group (n = 229; average age = 37 years); control group (n = 229; average age = 38 years) | Randomized, double blind, placebo controlled | - ↓ Duration of episodes of common colds  
- ↓ Number of days with fever during a common cold episode  
- ↑ Enhancement of cytotoxic plus T suppressor cells (CD8⁺) and a ↑ enhancement of T helper cells (CD4⁺) in probiotic-treated group |

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<table>
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<tr>
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<th>Study Design</th>
<th>Immune Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Winkler et al. (2005)</td>
<td><em>L. gasseri</em> PA 16/8 (4 × 10⁸ CFU/day), <em>B. longum</em> SP07/3 (5 × 10⁷ CFU/day) and <em>B. bifidum</em> MF 20/5 (5 × 10⁷ CFU/day) for 14 days during the winter/spring</td>
<td>Healthy adults; age range 18–70 years; n = 60 in test group; n = 60 in placebo group</td>
<td>Randomized, double blind, placebo controlled</td>
<td>• ↑ Total leukocyte, lymphocyte (CD45⁺), T-cell (CD45⁺CD3⁺), Th cell (CD45⁺CD3⁺CD4⁺), T cytotoxic plus suppressor cell (CD45⁺CD3⁺CD8⁺), and monocyte cell counts&lt;br&gt;• No effect on:&lt;br&gt;  • T-cell activation (measured by CD69 expression in PHA stimulated whole blood)&lt;br&gt;  • Phagocytic activity of monocytes or granulocytes&lt;br&gt;  • B cell (CD45⁺CD19⁺), NK cell (CD45⁺CD56⁺), or granulocyte cell counts</td>
</tr>
<tr>
<td>Morimoto et al. (2005)</td>
<td><em>L. casei</em> Shirota (LcS) drink (4 × 10¹⁰ live cells/80 ml) daily for 3 weeks</td>
<td>Male smokers of 20–60 years age (mean = 46.8); placebo n = 19; LcS group, n = 19</td>
<td>Randomized, double blind, placebo controlled</td>
<td>• The activity of NK cells was ↑ in LcS drinking smokers compared to placebo.&lt;br&gt;• No difference in proportion of NK cells.</td>
</tr>
<tr>
<td>Parra et al. (2004)</td>
<td><em>L. casei</em> DN114001 (2.85 × 10¹⁰–¹² CFU/day for 8 weeks)</td>
<td>Healthy adults; age 51–58 years; n = 23 in test group; n = 22 in control group</td>
<td>Randomized, double blind, placebo controlled</td>
<td>• ↑ Oxidative burst capacity of monocytes&lt;br&gt;• ↑ Tumoricidal activity of NK cells (lysis of K562 cells, at ratios of NK:K562 cells of 12:1 and 25:1)</td>
</tr>
<tr>
<td>Study</td>
<td>Intervention</td>
<td>Outcome</td>
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<tr>
<td>Marcos et al. (2004)</td>
<td>Yogurt cultures plus <em>L. casei</em> DN-114001 (Actimel) 100 ml volume daily for 6 weeks</td>
<td>- No effect on:</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>- Number of leukocytes, neutrophils, basophils, eosinophils, monocytes, or proportions of lymphocytes (CD19+ B cells or CD3+ T cells or CD3−CD16−CD56+ NK cells)</td>
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<tr>
<td></td>
<td></td>
<td>- Proportion of granulocytes or monocytes showing oxidative burst capacity</td>
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<td></td>
<td></td>
<td>- Intensity of oxidative burst capacity of granulocytes</td>
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</tbody>
</table>

The treatment period included 3 weeks before as well as 3-week duration of the examination period. The data were collected at the baseline before the start of intervention and at the end of intervention period.

- The mean anxiety increased significantly (*p* $<$ .05) over the 6-week study in all students.

- Significant (*p* $<$ .05) treatment effect on the mean change in the absolute number of lymphocytes during the 6-week study, which decreased in the control group.

- Significant treatment effect (*p* $<$ .05) on the change in absolute numbers of CD56 cells during the 6-week study. Mean absolute CD56 cells significantly (*p* $<$ .05) decreased in the control group while remaining similar in the treatment group.

(continued)
Table 22.1 Immune-Enhancing Effects of Probiotics (Continued)

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<tr>
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</table>
| Schultz et al. (2003)| *L. rhamnosus* GG (freeze-dried; $2 \times 10^9$ CFU/day for 5 weeks) | Healthy adults; mean age = 29.9 years (range 21–43 years); $n = 10$; no control group | Before and post-intervention | • No effect on CD4$^+$ T-cell proliferation (measured by ATP release) after stimulation with self and non-self whole fecal samples, although there was a trend to ↓. No effect after stimulation with *L. rhamnosus* GG.  
• ↑ CD4$^+$ T cell proliferation (measured by ATP release) after stimulation with self Bfg and self and non-self *E. coli*, and a nonsignificant ↓ was seen after stimulation with non-self Bfg.  
• ↓ TNF-α secretion by peripheral blood cells after stimulation with PHA, self and non-self fecal samples and self Bfg, but no effect with *L. rhamnosus* GG, non-self Bfg, self or non-self *E. coli*.  
• ↓ IL-6 secretion by peripheral blood cells after stimulation with *L. rhamnosus* GG, self fecal samples, self and non-self *E. coli* and PHA, and a trend for ↓ secretion after stimulation with self Bfg, but no effect with non-self fecal samples or non-self Bfg.  
• ↓ IFN-γ secretion by peripheral blood cells after stimulation with PHA and self-Bfg. |
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- **IL-10 production by peripheral blood cells after stimulation with non-self fecal samples and non-self Bfg, but no effect with *L. rhamnosus* GG, self fecal samples, self Bfg, self or non-self *E. coli* or PHA.**
- **No significant effects on IL-4 secretion by peripheral blood cells.**

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**Collins et al. (2002)**

*L. salivarius* UCC118 given in either fresh pasteurized milk (**10^10** CFU/day) or fermented milk (**10^10** CFU/day) for 21 days

Healthy adults; age 20–65 years; *n* = 20 in pasteurized milk group; *n* = 20 in control pasteurized milk group; *n* = 20 in fermented milk group; *n* = 20 in control fermented milk group

Randomized, double blind, placebo controlled

- **Regarding the systemic antibody response,** there was no effect on number of subjects exhibiting a UCC118-specific serum IgG response, or UCC118-specific serum IgG titers in those who were serologically positive at baseline.
- **Regarding the mucosal antibody response,** † number of subjects with positive shifts from baseline of **>75%** in salivary IgA against UCC118 at weeks 3 and 6 in those receiving the probiotic either by fermented milk or fresh milk (when analyzed by subgroups, this was also significant for the probiotic fermented milk group, but not for the fresh milk group).
- **† Granulocyte phagocytic activity in fermented milk group.**

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<tr>
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</tr>
</thead>
</table>
|                  |                                          | Healthy adults; aged 22–55 years; n = 20; no control   | Before and post-intervention                           | - No effect on:  
  - Granulocyte phagocytic activity in fresh milk group.  
  - Monocyte phagocytic activity in either group.  
  - Serum IL-1α, IL-1β, IL-4, soluble IL-2 receptor, soluble IL-6 receptor, TNF-α, and IFN-γ concentrations.  
  - ↑ % of CD4+ T cells (especially in those with the highest *L. paracasei* concentrations in the stools) after 2 weeks, but values ↓ again after 5 weeks  
  - ↓ % of CD54/ICAM-1 positive lymphocytes after 2 and 5 weeks  
  - ↑ Phagocytic activity of monocytes and granulocytes after 5 weeks  
  - No effect on:  
    - Oxidative burst activity of granulocytes  
    - % of CD3, CD19, CD8, CD16 and 56, CD8+ CD57, and CD122                                                                                     |
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<tr>
<th>Study</th>
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<th>Participants</th>
<th>Intervention Details</th>
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</table>
| Sheih et al. (2001) | *L. rhamnosus* HN001 (10⁹ CFU/g, consumed 50 g/day for 3 weeks, given in either low-fat milk, or lactose-hydrolyzed low-fat milk) | Healthy adults; middle aged, median age 63.5 years (range 44–80 years); n = 25 in low-fat milk group; n = 27 in lactose-hydrolyzed low-fat milk group | A three stage, pre–post intervention trial, spanning 9 weeks | • ↑ Relative proportion of polymorphonuclear cells showing phagocytic activity in both groups  
  • ↑ Relative level of NK cell tumor-killing activity in both groups (this was increased even further after a washout period of 3 weeks in the low-fat milk group)  
  • There were no significant differences between groups regarding the changes |
| Gill et al. (2001a) | *L. rhamnosus* HN001 (5 × 10⁹ organisms/day) for 3 weeks         | Healthy elderly adults; n = 13; acted as own controls; median age of females = 69.5 years (range 65–73 years); median age of males = 70 years (range 66–85 years) | Before and post-intervention                                                            | • ↑ Phagocytic capacity of monocytes and polymorphonuclear cells  
  • ↑ NK-cell tumoricidal activity (% of K562 tumor cells killed) |
| Gill et al. (2001c) | *L. rhamnosus* HN001 (5 × 10¹⁰ CFU/day) or *B. lactis* HN019 (5 × 10⁹ CFU/day) for 3 weeks | Healthy elderly adults; median age 69.5 years (range 60–84 years); n = 13 in *L. rhamnosus* group; n = 14 in *B. lactis* group; acted as own control | A three stage, pre–post intervention trial, spanning 9 weeks | • Both diets:  
  • ↑ Proportion of peripheral blood cells labeled singly for CD56⁺ (nonthymic NK cells)  
  • ↑ Tumoricidal activity of blood mononuclear cells (by 101% in *L. rhamnosus* group and 62% in *B. lactis* group)  
  • Subjects aged over 70 years had greater improvements in NK cell activity than those aged less than 70 years  
  • Proportions of dual-labeled T/NK cells (CD3⁺/CD56⁺) did not change |
### Table 22.1 Immune-Enhancing Effects of Probiotics (Continued)

<table>
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<tr>
<th>Reference</th>
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</table>
| Gill et al. (2001d)        | *B. lactis* HN019 (5 × 10^10 organisms/day or 5 × 10^9 organisms/day for 3 weeks) | Elderly adults; median age 69 years (range 63–84 years); independent living; n = 15 in typical dose group; n = 14 in low-dose group | A three-stage, pre–post intervention trial, spanning nine weeks | • There were no differences between the two groups regarding the immune variables measured, but within both groups there were time-dependent treatment effects regarding:  
  • ↑ Proportion of mononuclear leukocytes staining positively for CD3^+ (T cells), CD4^+ (Th cells), CD25^+ (IL-2 receptor), and CD56^+ (NK cells)  
  • ↑ % of mononuclear and PMN cells showing phagocytic activity (individuals with the poorest pre-intervention immunity, and who received the higher dose of *B. lactis* HN019, had a significantly greater relative increase in these values compared with those who had adequate pre-intervention immunity)  
  • ↑ Tumoricidal activity of mononuclear cells against K562 cells  
  • No effect on proportion of mononuclear cells staining positively for CD8^+ (cytotoxic T cells), CD19^+ (B cells), or HLA-DR^+ (APC bearing MHC-II) |
<p>| Gill and Rutherfurd (2001) | <em>L. rhamnosus</em> HN001 DR20™ (5 × 10^10 CFU/day for 3 weeks) | Healthy elderly; aged 62–77 years; n = 13 in test group; acted as own control | A three stage, pre–post intervention trial, spanning nine weeks | ↑ Phagocytic activity of peripheral blood PMN cells and mononuclear (monocyte) cells                                                  |</p>
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<th>Bacterium and Treatment</th>
<th>Participants</th>
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<th>Results</th>
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</table>
| Nagao et al. (2000) | *L. casei* strain Shirota (4 × 10^{10} live cells/ day for 3 weeks) | Adults; mean age 32.67 years (range 20–40 years); healthy but with low levels of NK activity (<45% cytotoxicity); n = 9 in test group; n = 8 in control group | Randomized, placebo controlled | • ↑ NK activity in the test group.  
• The biggest effect was seen in the subjects who had the lowest levels to start with.  
• No effect on:  
  - Frequency of CD3<sup>+</sup>CD16<sup>+</sup>CD56<sup>+</sup> NK cells among blood mononuclear cells.  
  - Frequency of CD4<sup>+</sup> or CD8<sup>+</sup> cells among blood mononuclear cells.  
  - Serum IFN-γ concentration.  
  - Serum IFN-α could not be detected. |
| Chiang et al. (2000) | *B. lactis* HN019 (10<sup>9</sup> CFU/g, given in low-fat milk, or lactose hydrolyzed low-fat milk, consumed 50 g/day for 3 weeks) | Healthy adults; median age 60 years (range 41–81 years); n = 27 in low-fat milk group; n = 23 in lactose hydrolyzed low-fat milk group | Double-blind, three-stage before-and-after intervention trial spanning 9 weeks | • ↑ % PMN cell phagocytosis.  
• ↑ % NK cell tumor killing.  
• Both of these increases were greatest in the group who consumed the *B. lactis* in lactose-hydrolyzed low-fat milk (significantly so for NK cell lysis and nonsignificant for PMN cell phagocytosis). |
| Arunachalam et al. (2000) | *B. lactis* HN019 (3 × 10^{11} CFU/day for 6 weeks) | Elderly healthy adults; median age 69 years (range 60–83 years); n = 13 in test group; n = 12 in control group | Randomized, double blind, placebo controlled | • ↑ Production of IFN-γ by blood mononuclear cells.  
• ↑ Number of PMN cells showing phagocytic activity (this increase was still evident 6 weeks after ceasing supplementation). |

(continued)
### Table 22.1 Immune-Enhancing Effects of Probiotics (Continued)

<table>
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<th>Reference</th>
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</table>
| Donnet-Hughes et al. (1999)| *L. johnsonii* La1 (1.5 × 10⁹ CFU/day or 1.5 × 10⁸ CFU/day for 3 weeks) | Healthy adults; aged 21–57 years; n = 42 in total, over the control and two test groups | Randomized groups double blind, placebo controlled | • Both test and control groups showed increased bactericidal activity of polymorphonuclear cells, this increase was still evident 6 weeks after ceasing supplementation, and there was no difference between groups.  
• ↑ Phagocytic activity with 10⁹ CFU/day  
• ↑ Respiratory burst with 10⁹ CFU/day  
• No significant effect of 10⁸ CFU/day on phagocytic activity or respiratory burst |
| Spanhaak et al. (1998)     | *L. casei* Shirota (10⁹ CFU/ml, 300 ml/day for 4 weeks) | Healthy men; mean age = 55.8 years (range 40–65 years); n = 10 in test group; n = 10 in control group | Randomized groups double blind, placebo controlled | • No effect on:  
  • Percentages of T (CD3), Th (CD4⁺), T suppressor/cytotoxic (CD8⁺), NK (CD16 and 56), or B (CD19) cells  
  • NK cell activity (against K562 tumor cells)  
  • Stimulated blood mononuclear cell production of IFN-γ, IL-1β, or IL-2  
  • Percentage of phagocytosing neutrophils or percentage of neutrophils showing oxidative burst  
  • DTH reactions against *Candida, Diphtheria, Proteus, Streptococcus, tetanus, Trichophyton, tuberculin, and glycerin*  
  • Humoral parameters (IgM, IgG, IgA, IgD, IgE, C3, C4, or factor B) in blood |
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<table>
<thead>
<tr>
<th>Study</th>
<th>Probiotic</th>
<th>Participants</th>
<th>Intervention Details</th>
<th>Results</th>
</tr>
</thead>
</table>
| Marteau et al. (1997) | *L. johnsonni* La1 (3 × 10^10 CFU/day for 28 days) | Healthy volunteers; ages not given; n = 6 in test group; n = 6 in control group | Randomized groups | ↑ Serum IgA levels in test group (significant by Student’s t-test, but not by ANOVA)  
• No effect on:  
  • Serum IgM, IgG or total Ig levels  
  • Jejunal secretions of IgA, IgG, or IgM or specific antibodies against La1 after jejunal perfusion with control solution and La1 suspension |
| Jahn et al. (1996) | *Saccharomyces boulardii* (lyophilized; 15 × 10^9 viable cells/day for 21 days) | Healthy adults; median age = 33 years (range = 26–39 years); majority male; n = 12; no control group | Before and post-intervention | • In peripheral blood lymphocytes or intestinal lymphocytes there was no effect on % of T cells (CD3⁺), NK cells (CD16⁺CD57⁺), B cells (CD20⁺), CD8⁺ T cells, CD4⁺ T cells, CD4⁺CD8⁻ double-negative T cells, γδ⁺ T cells or CD4⁺/CD8⁺ ratio.  
• In peripheral blood lymphocytes or intestinal lymphocytes, there was no effect on CD4⁺ or CD8⁺ T cell expression of CD45RA, CD45RO, or CD29.  
• In blood or intestine, no effect on expression of HLA-DR.  
• ↑ Expression of the α-chain of the IL-2 receptor (CD25) on peripheral blood lymphocytes, but not intestinal lymphocytes, and this was only on CD4⁺ T cells, not CD8⁺ T cells. |

(continued)
Table 22.1 Immune-Enhancing Effects of Probiotics (Continued)

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<tr>
<th>Reference</th>
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</tr>
</thead>
</table>
| Fukushima et al. (1998) | *Bifdobacterium* containing follow-up formula    | Seven healthy Japanese children (15–31 months old) | Before and post-intervention analysis.            | • ↑ HML-1+ CD4+ T cell subpopulation of the intestine but not the peripheral blood, but no effect on expression of HML-1 on CD8+ T cells or peripheral blood lymphocytes.  
• No effect on IgA content in small intestinal perfusate. |
<p>| Kishi et al. (1996)    | <em>L. brevis</em> subsp. <em>coagulans</em> (group 2 = 1.5 × 10^8, group 3 = 3 × 10^8, group 4 = 6 × 10^8 bacteria/day, and group 5 = 3 × 10^8 heat-killed bacteria/day, for 4 weeks) | Healthy subjects; n = 60; mean age = 35 years; n = 12 in control group (group 1); n = 12 in group 2; n = 12 in group 3; n = 12 in group 4; n = 12 in group 5 | Randomized groups with placebo controlled | • Statistically significant increase in IFN-α production at 2 weeks (p &lt; .05) and at 4 weeks (p &lt; .05) in the group receiving 600 million bacteria per day and at 4 weeks (p &lt; .05) in the group receiving 300 million bacteria per day. |</p>
<table>
<thead>
<tr>
<th>Study</th>
<th>Probiotics Supplemented</th>
<th>Human Subjects</th>
<th>Study Design</th>
<th>Outcomes</th>
</tr>
</thead>
</table>
| Schiffrin et al. (1995, 1997) | *L. acidophilus* La1 (7 × 10^{10} CFU/day) or *B. bifidum* Bb12 (1 × 10^{10} CFU/day) for 3 weeks | Healthy adults; mean age = 36.9 years (range 23–62 years); n = 28; one group supplemented with *L. acidophilus*; one group supplemented with *B. bifidum*; no control | Randomized groups before and post intervention | • No effect on:  
• Consumption of heat-killed bacteria on IFN-α production.  
• Level of 2-5A synthetase activity measured in whole blood.  
• ↑ Global phagocytic activity of blood phagocytes (granulocytes and monocytes) after consumption of both probiotics. This effect was more pronounced in granulocytes than monocytes.  
• No effect on proportions of blood lymphocyte subsets-total T cells (CD3⁺), activated T cells (CD3⁺ HLA-DR⁺), B cells (CD19⁺), Th cells (CD3⁺CD4⁺), suppressor cytotoxic T cells (CD3⁺CD8⁺), NK cells (CD3⁻CD16⁻CD56⁻). |
The PMNs exhibited significantly greater improvement in phagocytic capacity compared with monocytes. The increases in phagocytic activity were dose dependent (Donnet-Hughes et al. 1999) and were maintained for several weeks after cessation of probiotic intake (Schiff in et al. 1995; Gill et al. 2001a, 2001d). In another study, Lactobacillus GG was found to induce activation of neutrophils (increased the expression of phagocytosis receptors CR1, CR3, FcγRI, and FcαR) in healthy subjects, but inhibit activation of neutrophils in milk-hypersensitive subjects following a milk challenge (Pelto et al. 1998). Enhanced oxidative burst or microbicidal capacity of PMN cells in subjects fed probiotics or yogurt has also been demonstrated (Arunachalam et al. 2000; Mikes et al. 1995; Parra et al. 2004).

Aging is associated with decline in immune capacity and enhanced susceptibility to infectious diseases and cancers (Gill et al. 2001b). Several studies have shown that probiotic intake is able to restore the age-related decline in phagocytic cell function (Gill 2003). Aged subjects fed milk containing L. rhamnosus HN001 or B. lactis HN019 for 3–6 weeks exhibited significantly higher phagocytic activity than subjects fed milk without probiotics (Arunachalam et al. 2000; Gill et al. 2001a, 2001b; Gill and Rutherfurd 2001; Sheih et al. 2001). A recent study (Ibrahim et al. 2010) involving elderly subjects (median age 86 years) given probiotic cheese (10⁹ colony-forming units [CFU]/day of L. rhamnosus HN001 and L. acidophilus NCFM for 4 weeks) also reported significantly enhanced granulocyte and monocyte phagocytic activity (p < .001) as well as the ex vivo cytotoxicity of natural killer (NK) cells (p = .05). Importantly, subjects with relatively poor preintervention immunity status consistently showed greater improvement in phagocytic cell function than subjects with adequate preintervention immune function (Gill et al. 2001c). Furthermore, enhancement in phagocytic capacity was also age related, with subjects older than 70 years exhibiting significantly greater improvements in immune function than those under 70 years (Gill et al. 2001a, 2001b; Gill and Rutherfurd 2001). It is important to note, however, that several studies have found no effect of probiotic intake on natural immune function (Spanhaak et al. 1998). Whether this has been due to the poor immunostimulatory ability of the probiotic strains used, suboptimal dosing, probiotic viability, or some other reason is not known. Strain- and dose-dependent differences in the ability of LAB to modulate immune function are well documented (Donnet-Hughes et al. 1999; Gill 1998).

An impaired immune capacity in students under examination stress is well documented. Marcos et al. (2004) investigated the effect of consumption of yogurt cultures plus L. casei DN-114001 (Actimel, 100 ml) daily for 6 weeks on the immune status in students appearing for exams. The results showed a significant (p < .05) treatment effect on the absolute number of lymphocytes and CD56 cells during the 6-week study. These results clearly demonstrate that the consumption of probiotics can significantly augment the poorer innate immune response in healthy individuals, indicating their usefulness for prophylactic purposes.

Research is limited in infants and children due to invasiveness of the assays involved. In a recent study in infants, Taylor et al. (2006) investigated the effect of probiotic L. acidophilus LAVRI-A1 (Lafti L10) administration on markers of innate immunity in 0–6-month-old infants. The results showed no difference in cytokine production by blood Mononuclear cells following stimulation with Pansorbin or lipopolysaccharide (LPS). Also, no differences were found in the antigen-presenting capacity of immunocompetent cells in these infants. Thus, probiotic supplementation to infants did not alter early innate immune responses. Whether the lack of effect was due to the immunomodulatory capacity of the probiotics strain used, immune parameters measured, timing of sampling or some other reason is not clear. Further trials, especially employing probiotics with known immunomodulatory effects, are needed to confirm these observations.
22.2.2.2 NK Cell Activity

The effect of probiotics intake on NK cell activity has been the focus of many studies (Table 22.1). Ortiz-Andrellucchi and co-workers (2008) examined immunological changes in recently delivered and lactating women during first 6 weeks of lactation following supplementation with milk fermented with L. casei DN114001. They reported a significant ($p = .026$) increase in NK cells and a nonsignificant increase of T and B lymphocytes during the puerperium in subjects given milk fermented with L. casei. Morimoto et al. (2005) showed that the consumption of L. casei Shirota (LcS) drink ($4 \times 10^{10}$ live cells/80 ml) was also effective at significantly ($p = .02$) increasing the NK cell activity in habitual smokers (20–60 years age). Takeda and Okumura (2007) also observed significant enhancement of NK cell activity in healthy middle aged (30–45 years old) adults with relatively low natural levels of NK cell activity, following supplementation with the same probiotics. Augmentation of NK cell activity (ex vivo) and increases in the percentage of NK cells in the peripheral blood in healthy subjects following regular consumption of yogurt or milk or fermented drink containing probiotics has also been demonstrated by others (Gill et al. 2001a; Chiang et al. 2000; Sheih et al. 2001; Olivares et al. 2006a; Takeda et al. 2006; Kim et al. 2006; Makino et al. 2010; Sierra et al. 2010). Interestingly, as with phagocytic activity, improvements in NK cell function in the elderly subjects, following intake of probiotics, was significantly correlated with age (Gill et al. 2001d).

22.2.2.3 Acquired Immunity

The acquired immunity comprises antibody- and cell-mediated responses and is characterized by its specificity and memory. The best evidence for the effect of probiotics on acquired immune responses comes from studies with viral and bacterial vaccines (Table 22.2). The effects of probiotics supplementation on pathogen-specific immune responses, following natural infections with bacterial and viral pathogens, are discussed in the later sections.

In a randomized, double-blind, placebo-controlled study, subjects given yogurt containing L. rhamnosus and L. paracasei had significantly higher virus-neutralizing antibody responses (mainly IgA), following vaccination with live attenuated polioviruses, compared with subjects given placebo (chemically acidified milk). The levels of polio-specific serum IgG and IgA in volunteers consuming yogurt were also significantly increased (de Vrese et al. 2001). In a subsequent study, de Vrese et al. (2005) examined the efficacy of a polio vaccine in healthy adults (20–30 years old) given probiotics L. rhamnosus GG ($10^{10}$/day) or L. paracasei subsp. paracasei CRL431($10^{10}$/day) for 5 weeks; the subjects were vaccinated against polio serotypes 1, 2, and 3 after 8 days into the study. Vaccine induced titer increased in both probiotic groups against all serotypes. Significant IgA titer against serotype 1 was found in LGG group. On the other hand, IgM titers against serotype 2 and 3 were higher in CRL431 probiotic group. However, West et al. (2008) found no effect on antibody response to polio, when administered in combination with DTaP and Hib conjugate vaccines, in healthy, full-term infants fed L. paracasei subsp. paracasei strain F19. Again the vaccination regimen (including the age group of the subjects) and/or probiotic strain could be a cause for the lack of effect. More recently Soh et al. (2010) examined antibody responses to hepatitis B vaccine (HepB) and DTPa-HepB in infants administered probiotics or placebo. Significant improvements in antibody responses to HepB surface antigens in subjects receiving probiotics and monovalent doses of HepB vaccine and DTPa-HepB, but not after three monovalent doses of HepB vaccine were observed. These results indicate a vaccine schedule-dependent immunostimulatory effect of probiotics.
<table>
<thead>
<tr>
<th>Reference</th>
<th>Probiotic</th>
<th>Vaccine</th>
<th>Study Population</th>
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<th>Immune Effect</th>
<th>Remarks</th>
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<tbody>
<tr>
<td>Perez et al.</td>
<td>Low-fat milk fermented by (S.) (thermophilus) (control product) or low-fat milk fermented by (S.) (thermophilus) and (L.) (casei) (test product)</td>
<td>DTP-Hib vaccine or a 23 valent anti-pneumococcal vaccine</td>
<td>Children (a total of 162 subjects) with a high index of natural exposure to microorganisms</td>
<td>Randomized, double blind, and placebo controlled</td>
<td>Rate of immunoglobulin and isoagglutinin acquisition was similar in both groups. There was no difference between groups in antibody levels neither before nor after vaccination.</td>
<td>Days of fever and number of episodes of infection were not statistically different in either group</td>
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<tr>
<td>Soh et al.</td>
<td>Probiotics or placebo</td>
<td>Monovalent doses of HepB vaccine at 0, 1 month and a DTPa-HepB combination vaccine.</td>
<td>Infants (a total of 202 subjects divided into two studies, one receiving monovalent doses of HepB and probiotic ((n = 29)) or placebo ((n = 28)) and another receiving three monovalent doses of HepB and probiotic ((n = 77)) or placebo ((n = 68))</td>
<td>Two studies, randomized, double blind, and placebo controlled</td>
<td>Probiotic supplementation improved HepB surface antibody responses in subjects receiving monovalent doses of HepB vaccine at 0, 1 month and a DTPa-HepB combination vaccine at 6 months (placebo: 187.97 [180.70–195.24], probiotic: 345.70 [339.41–351.99] mIU/ml) (p = .069), but not those who received three monovalent</td>
<td>Not described</td>
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<tr>
<td>Study</td>
<td>Intervention</td>
<td>Outcome</td>
<td>Results</td>
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<td>Boge et al. (2009)</td>
<td>Fermented dairy drink, containing the probiotic strain <em>L. casei</em> DN-114 001 and yogurt ferments (Actimel), or a nonfermented control dairy product twice daily for a period of 7 weeks (pilot) or 13 weeks (confirmatory)</td>
<td>Three influenza viral strains composing the vaccine H1N1, H3N2, and B. Vaccination was performed after 4 weeks into the study.</td>
<td>In the pilot study, the influenza-specific antibody titers increased after vaccination, being consistently higher in the probiotic product group compared with the control group under product consumption. In the confirmatory study, titers against the B strain increased significantly more in the probiotic group than in the control group at 3, 6, and 9 weeks post vaccination under product consumption ($p = .020$).</td>
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<th>Reference</th>
<th>Probiotic</th>
<th>Vaccine</th>
<th>Study Population</th>
<th>Study Design</th>
<th>Immune Effect</th>
<th>Remarks</th>
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<tbody>
<tr>
<td>Paineau et al. (2008)</td>
<td>Seven probiotic strains belonging to genera <em>Lactobacillus</em> and <em>Bifidobacterium</em> were tested separately. Each active capsule contained $1 \times 10^{10}$ CFU of bacteria or maltodextrin (placebo)</td>
<td>Vaccine composed of inactivated <em>Vibrio cholerae</em> antigenic fractions (Dukorals; SBL Vaccin AB, Stockholm, Sweden). It contains fractions from three strains of <em>V. cholerae</em> (Ogawa, Inaba, and Inaba Eltor) and recombinant cholera toxin B; one vaccine dose consists of ca. $10^{11}$ vibrons. The dosage was two capsules per day for 21 days.</td>
<td>Eighty-three healthy volunteers aged 18–62 years</td>
<td>Randomized, double-blind, and placebo-controlled studies</td>
<td>Between day 0 and day 21, IgG increased in two probiotic groups, e.g., <em>Bifidobacterium lactis</em> Bl-04 and <em>L. acidophilus</em> La-14, compared with controls ($p = .01$). Trends toward significant changes in immunoglobulin serum concentrations compared with controls ($p &lt; .1$) were found for six out of the seven probiotic strains.</td>
<td>Not described</td>
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<tr>
<th>Study (Year)</th>
<th>Probiotic Treatment</th>
<th>Vaccination Schedule</th>
<th>Age, Health, Group Size</th>
<th>Study Design</th>
<th>Summary</th>
<th>Additional Notes</th>
</tr>
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<tbody>
<tr>
<td>West et al. (2008)</td>
<td>Placebo or <em>L. paracasei</em> subsp. <em>paracasei</em> strain F19 (LF19) (1 × 10^9 CFU/serving of cereal, consumed on average just less than one serving/day, from 4–13 months of age)</td>
<td>Immunized with DTaP (diphtheria, tetanus toxoid and acellular pertussis), polio and Hib conjugate vaccines at 3, 5.5, and 12 months</td>
<td>Healthy, full term infants; <em>n</em> = 84 in test group; <em>n</em> = 87 in control group</td>
<td>Randomized, double-blind, and placebo-controlled studies</td>
<td>No effect on IgG response to diphtheria, tetanus toxoid, and HibPS; however, when adjusted for breast feeding duration and colonization with LF19, LF19 ↑ IgG anti-diphtheria concentrations (most evident for infants who were breast-fed for &lt;6 months) There was an interaction between the intervention, time, and colonization with LF19 on IgG anti-tetanus concentrations.</td>
<td>Days with infectious symptoms did not differ between the groups. Days with antibiotic prescriptions were fewer in the LF19 group (<em>p</em> = .044).</td>
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<td>Olivares et al. (2007)</td>
<td><em>Lactobacillus fermentum</em> (CECT5716) (1 × 10^10 CFU/day) or placebo (methyl cellulose) 2 weeks before vaccination and 2 weeks after vaccination</td>
<td>Trivalent influenza vaccine (A/New Caledonia/20/99 [H1N1], A/Fujian/411/2002 [H3N2], B/Shanghai/361/2002 [B])</td>
<td>Healthy adults; vaccinated with influenza vaccine 2 weeks into the trial; mean age = 33 years (range = 22–56 years); <em>n</em> = 25 in test group; <em>n</em> = 25 in control group</td>
<td>Randomized, double blinded, placebo controlled</td>
<td>↑ % Th cells (CD4+) in blood at 4 weeks (also in control group) • ↑ % T cytotoxic cells (CD8+) in blood at 4 weeks (also in control group) The incidence of an influenza-like illness during 5 months after vaccination (October to February) was lower in the group consuming the probiotic bacteria.</td>
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<tr>
<td>Reference</td>
<td>Probiotic</td>
<td>Vaccine</td>
<td>Study Population</td>
<td>Study Design</td>
<td>Immune Effect</td>
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- ↑ % memory T cells (CD3⁺CD45RO⁺) in blood at week 2 and week 4 (also in control group)
- ↑ % NK cells (CD56⁺) in blood at 4 weeks
- ↑ IL-12 concentration in plasma before vaccination (2 weeks) and also at 4 weeks (also in control group at this time point)
- ↑ TNF-α concentrations in plasma at week 2 and week 4
- ↑ Total IgG and IgM in plasma
- ↑ Anti-influenza specific-IgA in serum
- No effect on:
  - % T cells (CD3⁺) in blood
<table>
<thead>
<tr>
<th>Study</th>
<th>Probiotic strain</th>
<th>Intervention</th>
<th>Participants</th>
<th>Outcomes</th>
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<tbody>
<tr>
<td>Taylor et al.</td>
<td><em>L. acidophilus</em> LAVRI-A1 (Lafti L10, 3 x 10^9/day for 6 months)</td>
<td>Tetanus vaccine at 2, 4 and 6 months of age</td>
<td>Newborn infants; at risk of allergy (due to parental allergy, on the maternal side); (n = 89 in test group; n = 89 in control group) Blood samples were available from n = 58 in test group; n = 60 in control group</td>
<td>Randomized controlled trial&lt;br&gt;- Tetanus vaccine responses:&lt;br&gt;- ↓ Frequency of IL-10 responses to tetanus toxin compared with placebo group (p = .03)&lt;br&gt;- No effect on magnitude of IFN-γ responses to tetanus toxin&lt;br&gt;- % B cells (CD19+) in blood&lt;br&gt;- IL-10 or IFN-γ concentrations in plasma (but a trend for an ↑ IFN-γ concentration at weeks 2 and 4)&lt;br&gt;- Total plasma IgA, or anti-influenza-specific IgG or IgM&lt;br&gt;Not described</td>
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<td>Reference</td>
<td>Probiotic</td>
<td>Vaccine</td>
<td>Study Population</td>
<td>Study Design</td>
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<td>Kukkonen et al. (2006)</td>
<td>• <em>L. rhamnosus</em> GG ATCC53103 (10 x 10⁹ CFU/day), <em>L. rhamnosus</em> LC705 (10 x 10⁹ CFU/day), <em>B. breve</em> Bbi99 (4 x 10⁹ CFU/day) and <em>P. freudenreichii</em> ssp. <em>shermani</em> JS (4 x 10⁹ CFU/day), plus galacto-oligosaccharides. • The mothers consumed the product for 4 weeks before delivery, and the infants consumed half this dose for 6 months after birth.</td>
<td>DTwP (diphtheria, tetanus and whole cell pertussis) vaccines at 3, 4, and 5 months of age and a Hib polysaccharide conjugate vaccine at 4 months of age; n = 47 in test group; n = 40 in control group</td>
<td>Newborn, full term infants at risk of allergy (at least one parent had allergic rhinitis, atopic eczema, or asthma)</td>
<td>Randomized, double-blind, placebo-controlled trial</td>
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</table>
| de Vrese et al. (2005) | *L. rhamnosus* GG ATCC 53103 (10^10/ day) or *L. paracasei* subsp *paracasei* CRL431 (10^10/ day) for 5 weeks | Vaccinated against polio 1, 2, and 3 after 8 days | Healthy adults; male; age 20–30 years; \(n = 21\) in LGG group; \(n = 21\) in CRL431 group; \(n = 25\) in control group | Randomized, double-blind and controlled | • ↑ Vaccine-induced titer increase in neutralizing antibodies for poliovirus serotype 1-, 2-, or 3-specific neutralizing antibodies in LGG and CRL431 groups (the increase being significant for serotypes 1 and 2 with LGG and for serotype 3 with CRL431).  
• ↑ IgA titer increase after vaccination (significant for poliovirus serotype 1 specific IgA with LGG).  
• ↑ Vaccination-induced increase in poliovirus serotype 2- and 3-specific IgM titer (significant for serotype 2 with CRL431). | Not described |
Table 22.2  Probiotics and Responsiveness to Vaccines (Continued)

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<tr>
<th>Reference</th>
<th>Probiotic</th>
<th>Vaccine</th>
<th>Study Population</th>
<th>Study Design</th>
<th>Immune Effect</th>
<th>Remarks</th>
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</table>
| Mullié et al. (2004)| B. breve C50 and S. thermophilus fermented infant formula (nonviable and dose not given), fed until 4 months of age. | Pentacoq® - Vaccines against diphtheria and tetanus toxoids, poliomyelitis virus, *Haemophilus influenzae* and *Bordetella pertussis* at 2, 3, and 4 months | Newborn, full-term infants; formula fed; n = 11 in test group; n = 9 in control group | Randomized, double blind, and controlled | • No significant effect on poliovirus serotype 1,2 or 3 specific IgG.  
• No effect on the increase in proportion of protected persons after vaccination (protective levels of neutralizing antibody titers). | Not described |

Pentacoq® - Vaccines against diphtheria and tetanus toxoids, poliomyelitis virus, *Haemophilus influenzae* and *Bordetella pertussis* at 2, 3, and 4 months

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<tr>
<th>Study</th>
<th>Probiotics and Human Immune Function</th>
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<tr>
<td>Fang et al. (2000)</td>
<td><em>Lactobacillus GG</em> (4 × 10^10 CFU/day) or <em>L. lactis</em> (3.4 × 10^10 CFU/day) for 7 days</td>
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<td>Attenuated <em>S. typhi</em> Ty21a oral Vivotif1 vaccine capsule (Swiss Serum and Vaccine Institute, Bern, Switzerland)</td>
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<td>A total of 30 healthy adult volunteers (females = 15, males = 15) aged from 20 to 50 years; LGG n = 10; <em>L. lactis</em> n = 10, and placebo n = 9</td>
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<td>Randomized, double-blind, and controlled</td>
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<td>• ↑ CR3 receptor expression on neutrophils in blood in <em>L. lactis</em> group compared with placebo or LGG groups</td>
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<td>• No effect of either probiotic on blood measurements of CR1, FcγRI, FcγRII or FcγRIII expression on neutrophils or monocytes, or CR3 expression on monocytes</td>
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<td>• Response to the vaccine regarding number of total IgA, IgG, or IgM secreting cells, or <em>S. typhi</em> Ty21a specific antibody-secreting cells, although there was a trend for an increase in <em>S. typhi</em> Ty21a specific IgA in the LGG group</td>
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<th>Reference</th>
<th>Probiotic</th>
<th>Vaccine</th>
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<th>Study Design</th>
<th>Immune Effect</th>
<th>Remarks</th>
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</table>
| Isolauri et al. (1995) | *L. casei* GG ATCC53103 (two doses of $5 \times 10^{10}$ CFU/day for 5 days) | Immunized with D × RRV rhesus rotavirus vaccine | Healthy infants; 2–5 months old; $n = 60$; included a control group | Randomized, double blind, and controlled | • ↑ Number of rotavirus-specific IgM-secreting cells in serum 8 days after vaccination  
• ↑ Rotavirus IgA seroconversion  
• There was no effect on:  
  • Total IgM, IgA, or IgG immunoglobulin secreting cells in blood  
  • IgM or IgG seroconversion  
  • No IgA specific rotavirus antibody-secreting cells were detected in serum of either group 8 days after vaccination  
  • Post vaccination antibody levels  
  • Lymphocyte proliferation | Not described |
| Link-Amster (1994) | *L. acidophilus* La1 and *Bifidobacteria* Bb12 (1 × 10^7-8 CFU/g, 375 g/ day for 3 weeks) | Administered attenuated *Salmonella typhi* Ty21a (to mimic enteropathogenic infection) | Healthy adult volunteers, age 19–59 years (mean age = 37.3 years); n = 16 in test group; n = 14 in control group | Randomized and controlled | • ↑ *S. typhi* Ty21a specific serum IgA titers in test group  
• ↑ *S. typhi* Ty21a specific salivary IgA in the control group  
• ↑ Total serum IgA in test group  
• ↑ IgA and IgG specific antibody titers to *S. enteriditis*, but no difference between groups  
• *E. coli* specific IgG antibody titers in test group after a washout period  
• No effect on:  
  • Total serum IgG  
  • Total salivary IgA  
  • *E. coli* specific IgG antibody titers after the supplementation period | Not described |
The effect of probiotics on responses to bacterial vaccines has also been the subject of many studies. Link-Amster et al. (1994) and He et al. (2000) reported significantly higher Salmonella-specific serum IgA antibody and IgA-secreting cell responses following vaccination in healthy adult subjects given probiotics (B. bifidum, L. acidophilus La1, Lactobacillus). Consistent with these observations, a trend toward increased anti-Salmonella IgA levels in healthy adult subjects given LGG and oral Salmonella vaccine was reported by Fang et al. (2000). Strain-dependent immune-adjuvant activity of seven probiotic strains administered along with Vibrio cholera vaccine (Ogawa, Inaba, and Inaba Eltor) has also been demonstrated (Paineau et al. 2008). In children with a high index of natural exposure to microorganisms, adjuvant action of milk fermented with S. thermophilus or S. thermophilus and L. casei were tested in combination with DTP-HiB vaccine or anti-pneumococcal vaccine. No improvement in antibody response was observed in any of the groups studied. It was suggested that the high natural rate of early microbial exposure in infants and children belonging to a low socioeconomic group, living in a “less hygienic environment,” may be responsible for the absence of an additional immune-stimulating effect in response to probiotics supplementation (Perez et al. 2010).

Together these studies suggest that, irrespective of the probiotic strain used as an immunomodulator, viral antigens (Rotavirus, Polio, hepatitis B, and Influenza) responded more consistently in all age groups studied (infants, children, adults, and elderly). However, for polio vaccine, the adjuvant action of probiotics appeared to be significant only when used alone rather than in combination with other vaccines (West et al. 2008). On the other hand, responsiveness to bacterial antigens (Salmonella, Vibrio, DTP-Hib) in infants and children was dependent on the probiotic strain as well as on the level of earlier exposure to microbes. The exact mechanisms by which probiotics augment specific immune responses is not known. It is likely that probiotics mediate these effects by up-regulating antigen recognition and effector cell functions. The ability of probiotics to up-regulate expression of pattern recognition receptors (e.g., TLRs), MHC-II molecules, and co-stimulatory molecules on immunocompetent cells and enhance production of immunoregulatory cytokines is well documented (Miettinen et al. 2008; Drakes et al. 2004; Smits et al. 2005).

22.2.2.4 Effect on Cytokine Production

Cytokines, by regulating cell-to-cell communication, play a central role in the initiation, maintenance, and resolution of both innate and acquired immune responses. For example, IL-12 and IL-18 stimulate interferon (IFN)-γ production by T, B, and NK cells and IFN-γ increases phagocytic capacity of macrophages, induces MHC1 and MHCII expression on immunocompetent cells, augments antitumor cytotoxicity, promotes T helper cell function, and improves immunogenicity of vaccines (Nussler and Thomson 1992). On the other hand, tumor necrosis factor (TNF)-α, together with IFN-γ, potentiates microbicidal and antitumor toxicity of macrophages. IFN-α confers protection on susceptible cells against viral invasion, and IL-2 stimulates the proliferation and differentiation of NK and B cells, and plays a role in the induction and regulation of T-cell-mediated immune responses (Gill 2003). IL-10 and transforming growth factor (TGF)-β are pivotal for restoring immunological homeostasis through the regulation of overactivated Th1/Th2 immune response.

Thus, to understand mechanisms by which probiotics influence the functioning of various immunocompetent cells, several studies have examined the effect of probiotics intake on cytokine production in vivo (serum/plasma) and ex vivo. For example, several studies have reported increased levels of IFN-γ, IFN-α, and IL-2 in human subjects fed yogurt and/or probiotics, compared with the control group (Kishi et al. 1996; Aattouri and Lemonnier 1997; Halpern et al.
Probiotics and Human Immune Function

1991; Meyer et al. 2006; De Simone et al. 1986; Solis Pereyra and Leennonier 1991; Wheeler et al. 1997). Increases in cord blood (CB) IFN-γ levels ($p = .026$) and TGF-β in early breast milk in mothers fed *B. lactis* HN019 and *L. rhamnosus* HN001 during pregnancy (Prescott et al. 2008) and elevated levels of IL-10 at 6 months in infants given a mixture of *Bifidobacterium breve* Bb99 or *Propionibacterium freudenreichii* ssp. *shermanii* (Marschan et al. 2008a,b) have also been reported. In a more recent study involving elderly subjects, supplementation with LGG together with oligofructose was found to increase the production of serum IL-1β, IL-6, and IL-8 (Amati et al. 2010). Ouwehand et al. (2008) reported a negative correlation between level of *Bifidobacterium* species in the feces and pro-inflammatory cytokine TNF-α and the regulatory cytokine IL-10 in serum samples; the presence of fecal *B. longum* and *B. animalis* correlated with reduced serum IL-10. Enhanced capacity of immunocompetent cells derived from probiotics fed subjects, compared with those from control subjects, to produce a range of cytokines, following mitogen stimulation *ex vivo*, has also been reported (Gill et al. 2009). Contrary to these observations, several studies have found little or no effect of probiotics on levels of various cytokines, including IFN-γ in healthy subjects (Nagao et al. 2000; Collins et al. 2002; Schultz et al. 2003; Olivares et al. 2006a; Takeda and Okumura 2007). The reported variation in the responses could be due to differences in the immune status of population groups studied, probiotics strain, as well as doses administered. Strain-dependent differences in the ability of probiotics to induce cytokine secretion from immunocompetent cells are well documented (Miettinen et al. 1998; Niers et al. 2005).

### 22.3 Probiotics-Induced Immunoenhancement and Disease Resistance

An optimally functioning immune system is pivotal for optimum health. Deficiencies or dysregulation of immune function are associated with enhanced predisposition to infectious, immunoinflammatory (allergies, inflammatory bowel disease [IBD], etc.), and autoimmune diseases and cancers. For example, increased susceptibility of specific population groups with less than adequate immune competence, such as immunocompromised individuals, children, and the elderly, to gastrointestinal and respiratory tract infections is well documented. Current strategies for the prevention and management of infectious diseases rely on the use of vaccines, antibiotics, and other antimicrobials. However, many of the vaccines are not fully protective, especially in vulnerable population groups such as the elderly, and the frequent use of antibiotics is associated with a range of side effects and the emergence of drug-resistant pathogens. Several well-designed studies have provided unequivocal evidence that specific strains of probiotics may be effective in the prevention and/or control of gastrointestinal, respiratory and urogenital tract infections, and allergic disorders. Emerging evidence also suggests that probiotics-induced immunomodulation may be centrally involved in mediating many of these beneficial effects. In the following section, we provide an overview of what is known to date regarding the relationship between immunomodulation and probiotic-mediated disease protection.

#### 22.3.1 Gastrointestinal Infections

Prophylactic and therapeutic efficacy of probiotics, as a single strain or a combination of several strains, against infectious diarrhea and antibiotic-associated diarrhea has been the focus of numerous randomized, double-blind, placebo-controlled studies and several systematic reviews
and meta-analyses (Szajewska and Mrukowicz 2001; Van Neil et al. 2002; McFarland 2006; Johnston et al. 2006; Sazawal et al. 2006; McFarland 2007). Despite significant heterogeneity between studies, these meta-analyses have concluded that specific probiotic strains are effective in shortening the duration of acute infectious diarrhea, risk of traveller’s diarrhea, and the incidence of antibiotic-associated and community-acquired diarrhea. For example, a meta-analysis of 34 masked, randomized, placebo-controlled trials by Sazawal et al. (2006) concluded that probiotics were effective in reducing antibiotic-associated diarrhea by 52%, the risk of travellers’ diarrhea by 8%, and that of acute diarrhea of diverse causes by 34%. It also found that probiotic treatment was more effective in children (57% reduction in risk) than in adults (26% reduction in risk). Furthermore, the protective effect did not vary significantly among the probiotic strains used.

Challenge infection studies in experimental animals have shown that probiotic-induced potentiation of both nonspecific and specific immune responses (Gill et al. 2001e; Shu et al. 2001; Cross 2002) plays an important role in protection against intestinal infections. Despite significant evidence for the ability of specific probiotics to enhance immune function in healthy subjects (Table 22.1), a direct evidence for this in humans is lacking. Only a few human studies to date have examined the effect of probiotic supplementation on clinical outcomes and immune function at the same time. Kaila et al. (1992) and Majamaa et al. (1995) reported that a reduction in the duration of acute infectious diarrhea in children, following administration of probiotics, was accompanied by a significant increase in the frequency of circulating IgG-, IgM-, and IgA-secreting cells during the acute phase and with enhanced specific (rotavirus-specific IgA antibody producing cells and anti-rotavirus serum IgA levels) and nonspecific immune responses during the convalescent phase. Furthermore, the greater efficacy of viable, compared with inactivated probiotics, against acute viral diarrhea was also reported to be due to their superior immunostimulatory capacity; infants receiving viable Lactobacillus GG exhibited higher anti-rotavirus serum IgA response and higher frequency of rotavirus-specific IgA-secreting cell response than subjects administered inactivated Lactobacillus GG (Kaila et al. 1995). Similarly, an association between reduced frequency of rotavirus shedding and enhanced titers of anti-rotavirus IgA in stool samples, following supplementation with B. breve has also been reported (Araki et al. 1999). It is important to note that in the studies cited above, increases in immune responses, following probiotic supplementation, have been modest and thus their relevance to clinical outcomes remains unclear. Further well-designed studies that examine the efficacy of probiotics against intestinal infections, together with their effect on immune system, are urgently needed to clarify the role of immune-mediated protection against gut infections.

22.3.2 Extraintestinal Infections

The ability of probiotics to enhance immune responses beyond the GIT has stimulated interest in examining the protective effects of probiotics against infections at extraintestinal sites. The major focus of these investigations has been on respiratory (especially upper respiratory tract infections [URTI]) and urinary tract infections because of their worldwide prevalence and high public health impact.

22.3.3 Respiratory Tract Infections

The effectiveness of probiotics against respiratory tract infections has been the focus of several randomized controlled trials over the recent years (Table 22.3). The results of these studies have been mixed due to the use of different probiotics/products, probiotic dose, study population, nature of
Table 22.3 Probiotics and the Prevention of Respiratory Tract Infections: Some Examples

<table>
<thead>
<tr>
<th>Reference</th>
<th>Intervention</th>
<th>Study Population</th>
<th>Study Design</th>
<th>Clinical Outcome</th>
<th>Immune Correlate</th>
</tr>
</thead>
</table>
| Cox et al. (2010)       | *Lactobacillus fermentum* VRI-003 (PCC; 1.26 × 10^{10} daily) over 4 months | 20 healthy elite male distance runners | DBPC cross-over design | Subjects receiving PCC had significantly fewer days with respiratory symptoms (30 versus 72; \( p < .001 \)) and less severe respiratory illness. | - No difference in IgA and IgA1 levels or IL-4 and IL-12 levels.  
- Whole-blood cultures from PCC group showed two-fold greater change in IFN-\( \gamma \) compared with placebo. |
| Merenstein et al. (2010) | Fermented dairy drink containing *L. casei* DN-114 001 (200 ml/day; 10^8 CFU/ml) or a matching placebo for 90 days | 638 children 3–6 years old in daycare/schools | DBPC | No significant difference in the incidence of URTI and LRTI between the groups | NR                                                                                                   |
| Kazuyoshi et al. (2010) | Subjects given a test food containing BB536 (10^{11} CFU) daily for 5 weeks (P1) and also immunized with influenza vaccine at week 3. Subjects then randomized to receive BB536 or a placebo for 14 weeks (P2). | 27 elderly subjects (mean age 86.7 ± 6.6 years) | DBPC | Subjects receiving BB536 during P2 had significantly lower incidence of influenza and fever compared with the placebo group. | BB536 group had exhibited significantly higher Neutrophil phagocytic activity and NK cell activity during P1. During P2, these responses declined in both groups but tended to remain higher in the BB536 group. |

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<tr>
<th>Reference</th>
<th>Intervention</th>
<th>Study Population</th>
<th>Study Design</th>
<th>Clinical Outcome</th>
<th>Immune Correlate</th>
</tr>
</thead>
</table>
| Hojsak et al. (2010)| \textit{Lactobacillus GG} (10^9 CFU/day) in 100 ml fermented milk or placebo for 3 months | 281 children attending day care centers | DBPC         | • Children receiving LGG had significantly reduced risk of URTI (RR 0.66), a reduced risk of URTI lasting longer than 3 days (RR 0.57) and a significantly lower number of days with respiratory symptoms ($p < .001$).  
  • No effect on lower respiratory tract infections. | NR               |
<p>| Leyer et al. (2010) | \textit{L. acidophilus NCFM, L. acidophilus NCFM + B. animalis subsp. lactis Bi-07} or placebo twice daily for 6 months | 326 children          | DBPC         | Single and combination probiotics reduced incidence of fever by 53% ($p = .0085$) and 72.5% ($p = .0009$), coughing incidence by 41.4% ($p = .027$) and 62.1% ($p = .005$), and rhinorrhea by 28.2% ($p = .68$) and 58.8% ($p = .03$). Antibiotic usage and the duration of fever, coughing, and rhinorrhea were also significantly reduced. | NR               |</p>
<table>
<thead>
<tr>
<th>Study</th>
<th>Intervention</th>
<th>Participants</th>
<th>Study Design</th>
<th>Summary</th>
<th>NR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hojsak et al. (2010)</td>
<td><em>Lactobacillus</em> GG (10⁹ CFU in 100 ml fermented milk) or a placebo</td>
<td>742 hospitalized children</td>
<td>DBPC</td>
<td>Significantly reduced risk for respiratory tract infections (RR 0.38) and episodes</td>
<td>NR</td>
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<td></td>
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<td>of respiratory tract infections that lasted &gt;3 days (RR 0.4)</td>
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<td>Sazawal et al. (2010)</td>
<td>Milk-containing oligosaccharides (2.4 g/day) and <em>B. lactis</em> HN019 (1.9 × 10⁷ CFU) or control milk for 12 months</td>
<td>624 children, 1–3 years of age</td>
<td>DBPC</td>
<td>Probiotics and prebiotic (PP) administration reduced incidence of pneumonia by 24%, acute respiratory tract infections by 35%. Compared with the placebo group, children receiving PP had 16% and 5% reduction in days with severe illness and high fever, respectively.</td>
<td>NR</td>
</tr>
<tr>
<td>Makino et al. (2010)</td>
<td>Yogurt fermented with <em>L. delbrueckii</em> spp. <em>bulgaricus</em> OLL1073R-1 (90 g/day) or milk (100 ml/day) for 8 or 12 weeks</td>
<td>57 (median age 74.5 years) and 85 (median age 67.7 years) healthy elderly subjects</td>
<td>Randomized trial</td>
<td>Subjects receiving probiotic yogurt had 2.6 times lower risk (RR 0.39; <em>p</em> = .019) of catching common cold than those receiving milk.</td>
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<td>Intake of probiotic yogurt was associated with increase of NK cell activity (<em>p</em> = .028)</td>
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(continued)
Table 22.3  Probiotics and the Prevention of Respiratory Tract Infections: Some Examples (Continued)

<table>
<thead>
<tr>
<th>Reference</th>
<th>Intervention</th>
<th>Study Population</th>
<th>Study Design</th>
<th>Clinical Outcome</th>
<th>Immune Correlate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cáceres et al. (2010)</td>
<td>Probiotic drink containing <em>L. rhamnosus</em> HN001 (10^{10} CFU/day) or a placebo for 3 months</td>
<td>398 children (1–5 years old) attending daycare centers</td>
<td>DBPC</td>
<td>No difference in the number and duration of the acute respiratory infections, the days of absenteeism or antibiotic usage between groups. Also, no differences between the groups in the detection rates of <em>Staphylococcus aureus</em>, <em>Streptococcus pneumoniae</em>, and <em>Streptococcus pyogenes</em> in the oropharyngeal samples.</td>
<td>Significant increase in the sIgA levels in the HN001 group, compared with the control (p = .048).</td>
</tr>
<tr>
<td>Rautava et al. (2009)</td>
<td>Infant formula supplemented with <em>Lactobacillus</em> GG and <em>B. lactis</em> Bb-12 or placebo daily until the age of 12 months</td>
<td>Infants requiring formula before the age of 2 months</td>
<td>DBPC</td>
<td>• Reduced incidence of otitis media (22% versus 50%) and use of antibiotics (31% versus 60%) in the probiotic group during first 7 months of life&lt;br&gt;• Also, reduced incidence of recurrent respiratory infections, during the first year of life, in the probiotic group compared with placebo (28% versus 55%)</td>
<td>NR</td>
</tr>
</tbody>
</table>


<table>
<thead>
<tr>
<th>Authors et al. (2009)</th>
<th>Fermented dairy drink containing <em>L. casei</em> DN-114001 for 3 months, followed by an additional 1 month follow-up</th>
<th>1072 free-living elderly (median age 76 years) volunteers</th>
<th>Multicenter, DBPC</th>
<th>Significant reduction in duration/episode and cumulative durations for all URTI (<em>p</em> &lt; .001 and rhinopharyngitis; <em>p</em> &lt; .001)</th>
<th>No difference in the immune blood parameters (oxidative burst activity of monocytes, cytotoxic activity, counts of blood NK cells, and levels of serum cytokines; <em>n</em> = 100)</th>
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<tbody>
<tr>
<td>Pregliasco et al. (2008)</td>
<td>A range of synbiotic preparations containing three to five strains of <em>Lactobacillus plantarum</em>, <em>Lactobacillus rhamnosus</em>, and <em>Bifidobacterium lactis</em>, lactoferrin and prebiotics such as either FOS (short-chain fructo-oligosaccharides) or GOS (galacto-oligosaccharides) or placebo were administered over three different winter seasons</td>
<td>721 healthy adults</td>
<td>DBPC</td>
<td>Synbiotic administration reduced the incidence (by 30–45% and severity of respiratory diseases (respiratory infection, flu, and cold) during cold season</td>
<td>NR</td>
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<tr>
<th>Reference</th>
<th>Intervention</th>
<th>Study Population</th>
<th>Study Design</th>
<th>Clinical Outcome</th>
<th>Immune Correlate</th>
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<tr>
<td>Luigi et al. (2007)</td>
<td><em>Bacillus clausii</em> or placebo for 3 months</td>
<td>80 children (3-6 years of age) with recurrent respiratory infections (RRI)</td>
<td>Randomized, single-blind, multi-center, two-arm parallel-group</td>
<td>Children treated with <em>B. clausii</em> had shorter duration of respiratory infections in comparison with the control group both during the treatment phase (11.7 days vs 14.37 days, <em>p</em> = .037) and the follow-up period (6.6 days vs 10.92 days, <em>p</em> = .049).</td>
<td>NR</td>
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<tr>
<td>Hatakka et al. (2007)</td>
<td><em>L. rhamnosus</em> GG and LC705, <em>Bifidobacterium breve</em> 99, and <em>Propionibacterium freudenreichii</em> JS (n = 155) or placebo (n = 154) daily for 24 weeks</td>
<td>309 otitis-prone children (10 months-6 years of age)</td>
<td>DBPC</td>
<td>Probiotics had no effect on the occurrence, or the recurrence of acute otitis media or the carriage of nasopharyngeal carriage of otitis pathogens.</td>
<td>NR</td>
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<tr>
<td>Tiollier et al. (2007)</td>
<td>Fermented milk with <em>L. casei</em> or placebo for 1 month (300 ml/daily)</td>
<td>47 healthy male army cadets (21 years of age)</td>
<td>DBPC</td>
<td>No difference in the incidence of respiratory tract infections, but significantly greater proportion of rhinopharyngitis in the probiotic group</td>
<td>After the combat course, IgA levels maintained in the probiotic group, but decreased in the placebo group.</td>
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<tr>
<td>Study</td>
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<td>Participants</td>
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<td>Outcome</td>
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<tr>
<td>Fukushima et al. (2007)</td>
<td>Fermented milk containing <em>L. johnsonii</em> LC1 or placebo</td>
<td>24 enterally fed in-patients, aged over 70 years</td>
<td>DBPC</td>
<td>Significant reduction in the percentage of days with infection and lower frequency of respiratory symptoms in the probiotic group</td>
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<td>• ↓TNF-α levels in blood</td>
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<td>• ↑Phagocytic activity of blood leukocytes in subjects whose initial levels were low in the LC1 group</td>
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<tr>
<td>Kekkonen et al. (2007)</td>
<td><em>Lactobacillus</em> GG or placebo for a 3-month training period</td>
<td>41 marathon runners</td>
<td>DBPC</td>
<td>No effect on the incidence of respiratory tract infections</td>
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<td>Puccio et al. (2007)</td>
<td>Experimental formula containing <em>B. longum</em> BL999 (2 × 10⁷ CFU/ml) and a mixture of galacto-oligosaccharides and fructo-oligosaccharides for 7 months</td>
<td>138 infants who were not breast fed after the 14th day after birth</td>
<td>Prospective, randomized, reference controlled, double blind</td>
<td>Trend toward fewer respiratory tract infections in the symbiotic group</td>
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<td>Cobo et al. (2006)</td>
<td><em>L. casei</em> (DN-114001) fermented milk (<em>n</em> = 142) or a placebo (<em>n</em> = 109) for 20 weeks to determine effect on infectious disorders</td>
<td>251 children, 3–12 years old</td>
<td>DBPC</td>
<td>Lower incidence of lower respiratory tract infections in the probiotic group (32% versus 49%, <em>p</em> &lt; .05)</td>
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<tr>
<th>Reference</th>
<th>Intervention</th>
<th>Study Population</th>
<th>Study Design</th>
<th>Clinical Outcome</th>
<th>Immune Correlate</th>
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<tbody>
<tr>
<td>de Vrese et al. (2005)</td>
<td><em>L. gasseri PA 16/8, B. longum SP 07/3</em> and <em>B. bifidum MF 20/5</em> + vitamins and minerals or placebo</td>
<td>479 healthy adults</td>
<td>DBPC</td>
<td>Reduction (0 &lt; 0.06) in total symptom score, duration of common cold (p = .05) and number of days with fever (p = .02) in the probiotic group</td>
<td>Significant increase in CD8(^+) and CD4(^+) cells in the probiotic-treated group</td>
</tr>
<tr>
<td>Winkler et al. (2005)</td>
<td>Probiotics (<em>Lactobacillus</em> and <em>Bifidobacterium</em> spp.) plus vitamins and minerals or placebo</td>
<td>477 healthy adults</td>
<td>DBPC</td>
<td>Reduction (p &lt; .07) in the incidence of respiratory tract infections, total symptom score (p = .12), and number of days with fever (p = .03) in the probiotic group</td>
<td>Significant increase in T-lymphocytes (including CD4(^+) and CD8(^+) cells) and monocytes</td>
</tr>
</tbody>
</table>
| Weizman et al. (2005) | Infant formula containing *B. lactis BB12*, *L. reuteri*, or placebo for 3 months | 201 infants 4–10 months of age | Multicenter, RDBPC | • Significantly fewer febrile episodes in probiotic groups.  
• Infants receiving *L. reuteri*, compared with *B. lactis* and control groups, had significantly fewer days with fever, clinical visits, child care absence, or antibiotic prescriptions. | NR |
<table>
<thead>
<tr>
<th>Study</th>
<th>Intervention</th>
<th>Participants</th>
<th>Design</th>
<th>Results</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tubelius et al. (2005)</td>
<td>L. reuteri or placebo</td>
<td>262 healthy adults</td>
<td>DBPC</td>
<td>Fewer subjects in the L. reuteri group reported sick-leave compared with the placebo group (10.6% versus 26.4, ( p &lt; .01 )); frequency of sick leave 0.4% vs. 0.9%, ( p &lt; .01 ). Among the shift workers, 33% reported sick leave compared with 0% in the probiotic group, ( p &lt; .005 ).</td>
<td>NR</td>
</tr>
<tr>
<td>Schrezenmeir et al. (2004)</td>
<td>Nutritional supplement with L. acidophilus and Bifidobacterium spp. (( 10^9 ) CFU/g) plus fructo-oligosaccharides (3.5 g) or without synbiotics or a fruit-flavored drink</td>
<td>129 acutely ill children, aged 1–6 years, receiving antibiotic therapy for bacterial infections (tonsillitis, pharyngitis, otitis, or bronchitis/mild pneumonia) not requiring hospitalization</td>
<td>Open-label</td>
<td>No significant difference in the incidence of bacterial infections between the groups</td>
<td>NR</td>
</tr>
<tr>
<td>Glück and Gebbers (2003)</td>
<td>Fermented milk drink containing Lactobacillus GG (ATCC 53103), Bifidobacterium sp B420, Lactobacillus acidophilus 145, and Streptococcus thermophilus; or standard yogurt</td>
<td>209 healthy adults</td>
<td>ORPC</td>
<td>Significant reduction (( p &lt; .001 )) in the number of potential pathogenic bacteria in nasal cavity in probiotic group</td>
<td>NR</td>
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<tr>
<th>Reference</th>
<th>Intervention</th>
<th>Study Population</th>
<th>Study Design</th>
<th>Clinical Outcome</th>
<th>Immune Correlate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Turchet et al. (2003)</td>
<td><em>L. casei</em> (DN-114001) fermented milk or a placebo for 3 weeks</td>
<td>360 free-living elderly subjects</td>
<td>ORC</td>
<td>No effect on the incidence of infections</td>
<td>NR</td>
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<td>Reduction (20%, <em>p</em> &lt; .05) in the duration of infection in probiotic group</td>
<td></td>
</tr>
<tr>
<td>Río et al. (2002)</td>
<td>Fermented milk containing <em>L. acidophilus</em> and <em>L. casei</em> (10^7–8/ml) or fluid milk control for three months during months-autumn to winter</td>
<td>100 normal or undernourished children aged 6–24 months</td>
<td>ORC</td>
<td>Reduction in the severity and incidence of respiratory tract infections</td>
<td>NR</td>
</tr>
<tr>
<td>Hatakka et al. (2001)</td>
<td>Milk with or without <em>Lactobacillus GG</em> for 7 months</td>
<td>571 healthy children aged 1–6 years</td>
<td>Multicenter DBPC</td>
<td>Reduction (17%) in the number of children having respiratory infections with complications and lower respiratory tract infections, and reduction (19%) in antibiotic treatment for respiratory infections</td>
<td>NR</td>
</tr>
<tr>
<td>Study</td>
<td>Probiotics</td>
<td>Participants</td>
<td>Study Design</td>
<td>Outcomes</td>
<td>Notes</td>
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<td>Lykova et al. (2001)</td>
<td>Bifidobacterin forte</td>
<td>46 hospitalized children; 33 with complicated forms of acute respiratory virus infection and 13 with vegetovascular dystonia</td>
<td>(comparison group)</td>
<td>Not reported</td>
<td>Normalization of an impaired interferon status of children with respiratory tract infections</td>
</tr>
<tr>
<td>Hatakka et al. (2001)</td>
<td>Milk with or without <em>Lactobacillus</em> GG ($n = 289$) or standard milk</td>
<td>571 day-care children (1–6 years old) in 18 day care centers</td>
<td>RDBPC</td>
<td>Children in LGG group had – 17% fewer respiratory tract infections, fewer days of absence from day care (16% ↓, $p &lt; .05$) and longer time without respiratory symptoms (5 versus 4 days, $p &lt; .05$) and 19% less antibiotic usage ($p &lt; .05$)</td>
<td>NR</td>
</tr>
<tr>
<td>Lykova et al. (2000)</td>
<td>Bifidobacterin forte</td>
<td>129 children with acute viral and bacterial infections of the respiratory tract</td>
<td></td>
<td>Normalization of intestinal microflora</td>
<td>Improvements in the indices of T- and B-cell immunity, NK cell activity, and IFN-γ producing capacity of blood leukocytes</td>
</tr>
</tbody>
</table>

Note: DBPC, double blind placebo controlled; NR, not reported; RDBPC, randomized double blind placebo controlled; ORPC, open randomized placebo controlled; ORC, open randomized controlled.
infectious agent, and clinical end points. There is strong evidence to suggest, however, that specific probiotics are effective in reducing the incidence, duration, and/or severity of respiratory infections. It has also been shown that probiotic-mediated protection against respiratory tract infections, especially URTI, is associated with enhancement of several immune responses. For example, Kazuyoshi et al. (2010) reported that a significantly lower incidence of influenza and fever in elderly subjects given BB536, compared with control subjects, was associated with significantly higher phagocytic cell function and NK cell activity. A link between increased NK cell activity and lower risk of catching cold in subjects given probiotic yogurt, compared with subjects given milk was reported by Makino et al. (2010). Similarly, increases in IgA levels, phagocytic activity, and T-cell numbers (CD4+ and CD8+), together with reduced incidence, duration, and/or severity of respiratory infections have been reported by others (de Verese et al. 2005; Winkler et al. 2005; Fukushima et al. 2007). Normalization of perturbed intestinal flora in children with acute viral and bacterial respiratory tract infections and enhancement of T- and B-cell function, and NK cell activity following administration with probiotics has also been reported (Lykova et al. 2000). Interferons (α and γ) play an important role in host protection, especially against intracellular pathogens/viral infections. Increased levels of IFN-γ in blood and the increased capacity of blood leukocytes to produce IFN-γ ex vivo in subjects administered probiotics is well documented (Table 22.1). Furthermore, respiratory tract infections impair the host’s ability to produce IFNs. Supplementation with probiotics was also found to be effective in restoring IFN-producing capacity of children with acute respiratory tract infections (Lykova et al. 2001).

Challenge infection studies in mice have shown that reduced lung pathology and enhanced pathogen lung clearance and survival rate in probiotic-fed animals are associated with up-regulation of the respiratory innate and specific immune responses (Alvarez et al. 2001; Racedo et al. 2006; Villena et al. 2008). Whether probiotics supplementation also influences respiratory innate and specific immune responses in humans remains unknown. However, the possibility that being a part of the common mucosal immune system, a small number of sensitized immune cells induced in the GIT may home to respiratory tract mucosa cannot be discounted (Macpherson et al. 2008).

22.3.4 Urogenital Infections

*Lactobacillus* species dominate the vaginal flora in healthy women. The depletion of lactobacilli is associated with increased susceptibility to urinary and vaginal tract infections. As a result, the use of probiotics to maintain and/or restore vaginal microbial balance and prevent and/or treat urogenital infections has been the focus of active research. The results of these studies have been highly variable. While a small number of studies have reported both therapeutic and prophylactic benefits of specific probiotic strains, others have found little or no benefit (Barrons and Tassone 2008; Reid 2008). Whether this has been simply due to differences in strains, dosages, or frequency of treatment used by various studies or some other reason is not clear. A range of mechanisms by which lactobacilli/probiotics mediate their protective effects, including immune stimulation, have been suggested. However, the direct evidence supporting a role for immune-mediated protection remains limited and requires further research.

22.3.5 Cancer

Despite compelling evidence for anticancer activity of probiotics in animal models, the evidence from humans is still very preliminary. A small number of small-scale human intervention studies
using biomarkers of colon cancer risk suggest that probiotics and synbiotics may reduce cancer risk. For example, Ishikawa et al. (2005) reported a significant reduction in adenomas with moderate or severe atypia after 4 years, in patients with resected adenomas and Rafter and colleagues (2007) reported reductions in colorectal proliferation and the capacity of fecal water to induce necrosis in colonic cells, and improvements in epithelial barrier function in polypectomized patients following supplementation with probiotics or synbiotics. Supplementation of synbiotics was also associated with increased production of IFN-γ in the cancer patients and suppression of IL-2 secretion by blood mononuclear cells in polypectomized patients. Other immune functions were not affected. Increases in the percentage of T helper cells and decreases in the percentage of T suppressor cells in patients with Dukes A colorectal cancer given *L. casei* Shirota have also been observed (Sawamura et al. 1994).

The effectiveness of probiotics in preventing the recurrence of superficial bladder cancer after transurethral resection has also been demonstrated in a number of studies (Aso et al. 1992; Aso et al. 1995; Ohashi et al. 2002). A recent study has further shown that supplementation with probiotic *L. casei* given in combination with epirubicin, an anticancer drug, was significantly more effective than the epirubicin alone in reducing the long-term recurrence of bladder tumors (Naito et al. 2008).

Several mechanisms, including immune stimulation, by which probiotics mediate anticancer activity have been proposed. However, the changes observed in cancer studies involving humans thus far have been relatively small, and their significance in protection is unclear. Significant increases in NK cell activity, phagocytic cell function, and T-cell responses observed in healthy subjects (Table 22.1), together with significant evidence from animal studies, however, supports a role for immune mechanisms in protection (Matsuzaki et al. 2007). Evidence from epidemiological studies that population with low NK cell activity have a higher risk of cancer than the populations with intermediate or high NK cell activity supports this assumption (Imai et al. 2000).

### 22.4 Probiotics and Immunoinflammatory Disorders

#### 22.4.1 Allergies

Allergic responses are associated with aggressive immune responses to harmless environmental antigens in genetically susceptible individuals, and are characterized by polarized Th2 immune response: increased IgE levels and recruitment and activation of eosinophils. A reduced or inappropriate microbial exposure early in life has been linked with increasing prevalence of allergic diseases in the industrialized world. At birth, the immune system of the newborn is polarized toward Th1 phenotype. Exposure to microbial antigens early in life is considered essential for the establishment of a balance between Th1/Th2 responses. As a result, the efficacy of probiotics in the prevention and/or treatment of allergic diseases, especially atopic eczema, has been the subject of numerous investigations in recent years. Results of these studies have been highly variable. Some studies have shown highly significant effects of probiotics (Kalliomäki et al. 2001, 2003; Wickens et al. 2008) while others have found little or no effect (Rautava et al. 2006; Kukkonen et al. 2007; Abrahamsson et al. 2007). For example, a meta-analysis of 10 randomized, controlled trial (involving 678 children with eczema) by Michail et al (2008) concluded that probiotics administration (single or mixed strains) was significantly more effective (*p < .01*) in reducing SCORAD severity index compared with subjects given no probiotics. Furthermore, probiotics were more effective in treating moderately severe atopic disease and the effect seemed to be greater in the IgE-sensitized
Subjects. Contrary to this, however, Boyle et al. (2009) from a systematic review of 12 studies (781 patients with eczema) concluded that probiotics administration had no significant effect on allergy symptoms and was effective in reducing SCORAD only in 5 of the 12 studies. The reasons for variable results in different studies remain poorly understood but are likely to include host and environmental factors as well as the strain and dose of probiotics used.

Similarly, the preventive effects of probiotics have also been subject of several reviews and meta-analyses. From a meta-analysis of five studies reporting the outcomes for 1477 subjects, Osborn and Sinn (2009) found that probiotics were significantly effective in reducing the risk of infant eczema (RR 0.82). However, when the analysis was restricted to studies reporting atopic eczema (confirmed by skin prick test or specific IgE), the findings were no longer significant. Studies reporting significant benefits used probiotic supplements containing *L. rhamnosus* and enrolled infants at high risk of allergy. No other benefits were reported for any other allergic disease or food hypersensitivity outcome.

The efficacy of probiotics against asthma and allergic rhinitis largely remains unproven, although some encouraging results for allergic rhinitis have been reported in recent years.

Increasing evidence from human and animal studies further show that probiotics mediate their antiallergy effects through the activation of Th2 counter-regulatory immune responses. For example, cytokines produced by Tr1 and Th3 regulatory cells (IL-10 and TGF-β, respectively) play a pivotal role in regulating polarized Th1 and Th2 responses. Th1 and Th2 cells are also known to possess counter-regulatory properties. Increased levels of TGF-β and IFN-γ in serum and/or breast milk, following supplementation with probiotics, in healthy and allergic subjects are well documented (Pessi et al. 2000; Prescott et al. 2005; Rautava et al. 2002). An enhanced ability of specific probiotics strains to induce regulatory cells that produce TGF-β and IL-10 and inhibit proliferation and cytokine production by T cells has also been reported (Di Giacinto et al. 2005; Smits et al. 2005). Contrary to this, however, Bottcher et al. (2008) reported that low, rather than high TGF-β levels in colostrum and milk were associated with reduced sensitization and reduced incidence of IgE-associated eczema following supplementation with probiotics. Other mechanisms such as reduced immunogenicity of potential allergens and strengthening of mucosal barrier through stimulation of sIgA production induced by probiotics have also been suggested to play a role.

### 22.4.2 Inflammatory Bowel Disease

IBD includes Crohn’s disease (CD), UC, and pouchitis. These conditions are characterized by a shift from tightly regulated intestinal immune response to uncontrolled immune cell activation and production of pro-inflammatory cytokines. UC is mainly restricted to the colon and rectum, whereas CD occurs as skip lesions in any region of the intestinal tract. Pouchitis is inflammation of the ileal reservoir in patients with ileal-pouch anal anastomosis (a surgical procedure commonly used for the management of UC). Little is known about the etiology of IBD. Several lines of evidence suggest that IBD results from an aberrant immune response, in genetically susceptible individuals, to members of the gut microbiota. The conditions are thought to be driven by T cells and are associated with increased production of pro-inflammatory cytokines such as TNF-α and IFN-γ. It has also been suggested that UC is associated with predominance of Th2-type responses, whereas CD is associated with Th1 polarization. A role for TH17 cells in the perpetuation of IBD has also been suggested. Differences in the composition of gut microbiota of healthy versus IBD patients and in the active and inactive stages of IBD in identical patients (Nishikawa et al. 2009) have also been reported. Since probiotics are known to possess the ability
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to restore gut microbial balance, stabilize gut mucosal barrier, and correct polarized Th1/Th2 immune responses, their efficacy in IBD has been the focus of several studies. However, only a few of these trials have been randomized-controlled trials. Also, most of these trials have been performed in relatively a small number of patients using different probiotics strains. The role of probiotics in IBD is also discussed in Chapter 23.

There is strong evidence that specific probiotics preparations are effective in inducing remission and reducing the recurrence rate of UC. A meta-analysis of 13 randomized studies concluded that probiotics, compared with placebo/nonprobiotic treatment, were significantly effective in improving the remission rate (1.35–2.0) and reducing the recurrence rate of UC (0.69) (Sang et al. 2010). Zigra et al. (2007) also showed that the safety and efficacy of probiotics in inducing remission of UC was similar to that observed for standard therapy.

There is also strong evidence for the effectiveness of probiotics, especially VSL#3 (a mixture of several probiotics strains) in preventing the initial attack of pouchitis, and in maintaining antibiotic-induced remission in patients with recurrent/refractory pouchitis (Vanderpool et al. 2008). A meta-analysis of five randomized, placebo-controlled clinical trials revealed an odds ratio of 0.04 (95% confidence interval 0.01–0.14) in favor of the treatment group (Elahi et al. 2008). Contrary to above studies, the efficacy of probiotics against CD remains unproven. The results of various studies have been heterogeneous and difficult to interpret.

The primary mechanisms by which probiotics mediate protection against IBD appears to be through the strengthening of gut mucosal barrier function, restoration of immunological homeostasis via up-regulation of immunoregulatory pathways and down-regulation of pro-inflammatory responses, and maintenance of gut microbial balance. For example, Ulisse et al. (2001) and Lammers et al. (2005) reported a reduction in pro-inflammatory cytokines (TNF-α, IL-1β, and IFN-γ) and an increase in IL-10 in intestinal tissues from subjects with pouchitis following treatment with probiotics. An association between expression of IL-10 and the prevention of flare-ups of chronic UC (Cui et al. 2004) and amelioration of inflammation in Helicobacter hepaticus–induced IBD in an animal model (Pena et al. 2005), following treatment with probiotics, has also been reported. A recent study has further shown that VSL#3 improves pouchitis disease activity index by increasing the number of mucosal regulatory T cells (Pronio et al. 2008).

22.4.3 Irritable Bowel Syndrome

IBS is a multisymptom gastrointestinal disorder of unknown etiology. Dysbiosis (alterations in the gut microbiota, small intestinal bacterial overgrowth), acute viral gastroenteritis, and altered inflammatory and immune state in the GIT have been suggested to play a role in the pathogenesis of IBS. An imbalance between IL-10 and IL-12 in patients with IBS has also been reported (O’Mahony et al. 2005).

There is some evidence to suggest that intake of probiotics may be beneficial in alleviating the symptoms of IBS (please see also Chapter 23). For example, a review of 14 randomized controlled trials involving IBS patients given different probiotics found that Bifidobacterium infantis 35624 was effective in significantly improving both global and individual symptoms of IBS. Other probiotics strains were found to be ineffective. Another systematic review by Moayyedi et al. (2010) found that probiotics treatment had a significant effect in reducing IBS symptoms with a number needed to treat of 4 (95% confidence interval 3–12.5).

The exact mechanisms by which probiotics mediate protection against IBS are not known. B. infantis and L. rhamnosus GR1 plus L. reuteri RC 14 were found to mediate their effects by decreasing IL-12 production and correcting IL-10/IL-12 imbalance (O’Mahony et al. 2005). Increased
frequency of T reg cells in IBS patients given probiotics has also been demonstrated (Lorea Baroja et al. 2008).

### 22.5 Mechanisms by Which Probiotics Mediate Their Immunomodulatory Effects

Sampling of probiotics from the GIT is primarily mediated by M cells, overlying organized lymphoid follicles, that deliver these to professional antigen-presenting cells (APCs; macrophages and dendritic cells) present in the subepithelial dome of Peyer’s patches and lymphoid follicles. The ability of dendritic cells to efficiently acquire antigens directly from the gut lumen has also been reported. After processing, APCs present antigen to B and T cells. Activation of Th1 cells results in augmentation of cell-mediated immunity, of Th2 cells in enhancement of antibody responses (especially IgE), and of T regulatory cells (Tr1 and/or Th3) in the dampening/correction of overactivated Th1/Th2 immune responses, associated with immunoinflammatory disorders such as IBD and allergy, respectively. It is the nature of cytokine secretion, phenotype, and state of activation of APCs that determines whether naïve T cells differentiate into T helper 1 (Th1), T-helper 2 (Th2), or T regulatory (Treg) cells. It has also been suggested that probiotics regulate polarized Th1/Th2 response through induction of T-cell anergy and IL-10–dependent induction of tolerance (Peppelenbosch and Ferreira 2009). Th1 cells also direct the differentiation of B cells to IgA-producing cells. M cells have also been shown to exhibit the propensity for sampling IgA-complexed antigens that further promotes IgA production. Signals from probiotic organisms are also transmitted to the epithelium and are involved in maintaining tissue and immune homeostasis. The molecular mechanisms by which probiotics or their products modulate T-cell activation and function, and the nature of probiotics-derived immunoactive components responsible for immunomodulatory action are not known. Recent studies examining in vivo transcriptomic responses, following supplementation with probiotics, has shown that different probiotics induce differential gene-regulatory networks and pathways in human intestinal mucosa and that a large person-to-person variation in transcriptome exists among individuals (van Baarlen et al. 2009).

### 22.6 Conclusion

The immunomodulatory effects of probiotics have been the subject of extensive research over the past 20 years. The results of these studies, although variable, have provided strong evidence that specific strains of probiotics are able to influence a wide array of immune functions in health and disease. In the healthy state, specific probiotics have been shown to enhance NK cell activity and phagocytic activity of macrophages and PMN cells, stimulate slgA production, improve immunogenicity of both viral and bacterial antigens/vaccines, and strengthen gut barrier function. Improvements have been shown to be of much greater magnitude in subjects with poor preintervention immune status compared with those with adequate immune function. Emerging evidence also suggests that probiotics-induced immune stimulation contributes to host protection against infectious disease. Perhaps, it is best illustrated by the ability specific probiotics strains to mediate protection against extraintestinal infections, especially the respiratory tract. This has been suggested to be due to the ability of probiotics to augment protective immune responses at distal mucosal sites as well systemic immunity. However, there is little direct evidence linking
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up-regulation of specific and nonspecific immune responses at distal mucosal sites and improvements in clinical end points in humans. Studies that concomitantly measure clinical end points and aspects of relevant immune functions/correlates are urgently required to clarify this.

In subjects with immunoinflammatory disorders (such as allergies and inflammatory bowel disease), prophylactic and/or therapeutic efficacy of some strains has also been demonstrated. There is evidence to suggest that probiotics mediate their beneficial effects through the induction of T regulatory cells and/or T-cell anergy. However, little is known about the probiotics-derived components or metabolites and the molecular mechanisms by which these immunoenhancing, anti-inflammatory, and immunoregulatory effects are mediated in health and disease.

It is important to note that the immunomodulatory effects of probiotics are strain specific and in most cases these effects have been demonstrated for only a small number of strains. The effective dose, duration, and frequency of treatment for various strains for various conditions in different population groups also remain to be determined. The effects of host genetics and the composition of gut microbiota on responsiveness to probiotics are also not known. Understanding of these is essential to identify more efficacious probiotics and to develop probiotics tailor-made to suit an individual’s genetics and microbiota. This area of research has the potential to minimize the worldwide impact of infectious and immunoinflammatory diseases, including metabolic disorders, that constitute the biggest public health challenge in the modern era.

References


Probiotics and Human Immune Function


Lactic Acid Bacteria: Microbiological and Functional Aspects


Probiotics and Human Immune Function


Chapter 23

Gastrointestinal Benefits of Probiotics—Clinical Evidence

Anna Lyra, Sampo Lahtinen, and Arthur C. Ouwehand

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23.1 Introduction

A wide range of health effects, including enhanced metabolism of dietary compounds, alleviation of disturbed bowel functions, improved resilience of the gastrointestinal (GI) microbiota, resistance against infections within and outside the GI tract, and prevention of allergies have been linked with the use of specific probiotic microbes. The findings are based on the potential etiological role of the gut microbiota in the pathogenesis of a number of clinical conditions and in the modulation of host immune functions, providing means for modulating the host immune responses both locally and systemically. The proposed and documented GI benefits of the probiotics are based on several clinical intervention studies with varying quality and methodology and on meta-analyses on the aforementioned (Table 23.1). The following chapter will discuss the outcomes of probiotic intervention studies on GI disorders and diseases.
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<sup>a</sup> The results are based on trials conducted with varying probiotic supplements not taking into account the strain specificity.

<sup>b</sup> Overview based on a systematic review without meta-analysis.
23.2 Diarrhea

Numerous clinical studies aiming at the treatment or prevention of an array of diarrheal diseases by the use of probiotics have been published (Allen et al. 2010; Wolvers et al. 2010). Most of these studies have been conducted using mixtures of several probiotic strains, individual Lactobacillus strains, or a probiotic yeast, Saccharomyces boulardii. Overall, the duration of diarrhea can be shortened by 1 day to up to 3 days by using specific probiotics (Allen et al. 2010). In children, shortening of the duration of rotavirus diarrhea by the administration of probiotic bacteria is well established (Szajewska and Mrukowicz 2001; Allen et al. 2004; Sazawal et al. 2006), and probiotics have been accepted and widely used as diarrheal therapy alongside rehydration (Guarino et al. 2008). Moreover, certain probiotics have the potential to play a part in solving the substantial health problem caused by recurrent diarrheal episodes in developing countries (Preidis et al. 2011). However, there is still a need for further community-based intervention trials especially in developing countries since most of the current clinical studies have been carried out in hospitals and clinics in developed countries (Sazawal et al. 2006; Preidis et al. 2011).

Antibiotic treatment substantially disrupts the balance of the intestinal microbiota (Dethlefsen et al. 2008) and may cause antibiotic-associated diarrhea (AAD) or increase the risk of Clostridium difficile associated diarrhea (CDAD) (Beaugerie et al. 2003; De La Cochetiere et al. 2008). Elderly subjects, subjects with underlying diseases, or otherwise frail individuals are especially prone to CDAD (Henrich et al. 2009). Due to the high prevalence and potentially detrimental impact of AAD and CDAD on health, the effort to discover efficient probiotics against these diseases has been substantial (McFarland 2009). According to a meta-analysis on Lactobacillus probiotics, lactobacilli can effectively prevent AAD in both children and adults (Kale-Pradhan et al. 2010). For example, a probiotic drink containing Lactobacillus casei DN-114 001, Lactobacillus bulgaricus, and Streptococcus thermophilus has been shown to decrease the risk of AAD and CDAD among elderly subjects by 22% and 17%, respectively (Hickson et al. 2007). In children, however, the effect of a Lactobacillus rhamnosus probiotic product, including three different strains (E/N, Oxy, and Pen), showed only a trend of decrease in the risk of AAD and no protective effect against CDAD (Ruszczynski et al. 2008). One shortcoming in probiotic intervention studies on AAD is the varying length or complete lack of follow-up after the antibiotic course; since AAD may occur with an up to 2 months delay after the antibiotic treatment, inadequate follow-up may result in erroneous interpretations of results (McFarland 2009).

The use of probiotics against traveler’s diarrhea is popular. However, the clinical evidence for the prevention of traveler’s diarrhea is still somewhat limited. Meta-analyses have suggested an overall benefit for probiotics, but the magnitude of effects appears to be modest. The current evidence is limited by the inadequate amount of interventions and variations in the protocols (Sazawal et al. 2006; McFarland 2007). The wide variety of the potential causes of traveler’s diarrhea and the difficulties of the volunteers in adhering to the study protocols during traveling bring additional challenges to the probiotic interventions targeting traveler’s diarrhea.

There are many causes of diarrhea, and probiotic studies targeting the same diarrhea subtype may use different criteria in subject recruitment and varying outcome measures (Allen et al. 2010), making joint conclusions on probiotics and diarrheal diseases difficult. Nevertheless, probiotics appear to be most effective in the prevention and treatment of rotavirus diarrhea and AAD, while the effect on travelers’ diarrhea needs further assessment.
23.3 Bowel Habits, Irritable Bowel Syndrome, and Constipation

Irregular bowel movements, often accompanied by abnormal stool form, are more common than regular bowel movements among Western adults (Heaton et al. 1992). Therefore, the potential market for probiotics that show a benefit on bowel habits among generally healthy adult subjects is substantial. Examples of such benefits include shortening of the colonic transit time (Marteau et al. 2002), increased stool frequency and volume (Olivares et al. 2006), and improved stool consistency (Larsen et al. 2006) and associated with that, improved ease of defecation.

Some individuals experience functional bowel symptoms in a recurrent fashion and can have a diagnosis of functional bowel disorders (FBDs), which by definition are devoid of structural and biochemical abnormalities (Longstreth et al. 2006). Consequently, intervention studies conducted with subjects found to have FBDs are applicable for the general population because i) the time requirements for diagnosis of FBDs are relatively loose (symptoms must have occurred for at least 6 months and have been present for 3 days a month during the preceding 3 months), ii) no defined etiological factors are known for FBDs, iii) no inflammatory or structural abnormalities explain FBD symptoms, and iv) the distinct symptoms encountered in FBDs are also common among the general population lacking GI-related diagnosed abnormalities. In addition, comparison between treatment groups is more efficient when the subjects are likely to experience bowel symptoms during the course of the intervention.

Irritable bowel syndrome (IBS) is an FBD associated with abdominal pain or discomfort, which is relieved by defecation or triggered in association to altered stool form or frequency and often associated with bloating and passage of mucus (Longstreth et al. 2006). Dysbiosis of the intestinal microbiota, including reduced numbers of the genus *Bifidobacterium* and symptomspecific alterations in lactobacilli levels, has been suspected as a possible etiological factor in IBS, although the causative nature of microbial dysbiosis has not yet been proven (Salonen et al. 2010). Moreover, disturbances of the gut microbiota caused by enteric infections and antibiotic courses are known to predispose to IBS (Mendall and Kumar 1998; Rodriguez and Ruigomez 1999). Thus, probiotics are an intriguing alternative for alleviation of the symptoms of IBS.

Several intervention studies on probiotics and IBS have been published, some of which fulfill the requirements of good quality randomized controlled trials (RCTs) with adequate subject numbers and intervention lengths. Since IBS is a symptom-based disorder that relies on the subject’s own sensation of bowel function, specific subject-filled questionnaires designed for IBS-related symptoms and quality of life are most often used as the outcome measures. The overall IBS symptoms and individual symptoms of abdominal pain, bloating, and flatulence are modestly but significantly improved with probiotics according to a recent meta-analysis (Hoveyda et al. 2009). As with all health targets, probiotic health effects in IBS are strain specific, and therefore meta-analyses in this field suffer from generalization arising from combining of health effects of different probiotic supplements (Dendukuri and Brophy 2007). In more recent RCTs the overall symptom score of IBS symptoms has been alleviated with several types of probiotics including *Escherichia coli*–based probiotic supplements (Enck et al. 2008; Enck et al. 2009), a bifidobacterial strain (Agrawal et al. 2008), and a multispecies probiotic supplement (Kajander et al. 2008). For quality of life assessments in IBS, the inadequate data published and variety of questionnaires used made combined analysis of different intervention studies impossible (Hoveyda et al. 2009). Moreover, the intestinal microbiota of IBS subjects has been shown to become more stable (Kajander et al. 2008) and to alter on phylotype level toward a putatively healthier type microbiota (Lyra et al. 2010) due to consumption of probiotics.
As to abdominal symptoms, IBS subjects form a heterogeneous group of people lacking a uniform etiological factor, and therefore it is possible that aiming at alleviating all symptoms associated with IBS with a single probiotic supplement might not be feasible. Different probiotic supplements could be more efficient within different subgroups of IBS subjects or during periods of defined symptoms for each subject as the prominent symptoms may vary with time. On the other hand, a functional GI symptom, such as pain or bloating, may be alleviated with a specific probiotic even among subjects with different diagnoses; in an intervention study with *Lactobacillus acidophilus* NCFM and *Bifidobacterium lactis* Bi-07, bloating was relieved among subjects with nonconstipation IBS, functional diarrhea, or functional bloating according to the Rome III criteria (Ringel et al. 2011). Moreover, consumption of probiotics during acute diarrheal infections and antibiotic courses could lower the risk of latter onset of IBS symptoms. An additional challenge in IBS intervention studies is the high placebo response rate, which, on the other hand, may increase the effectiveness of therapeutic use of any treatment devoid of adverse events (Pitz et al. 2005). Taken together, the treatment of IBS with probiotics holds great potential, but more large-scale RCTs of sufficient length are needed to further demonstrate the positive health effects of each probiotic supplement.

Constipation has traditionally been defined as less than three defecations per week, but not all people feel constipated with such a defecation frequency (Spiller and Thompson 2010) and others may feel constipated even though they have more frequent bowel movements. Thus, the effort needed to defecate and stool consistency are also taken into account in the more recent criteria for constipation (Longstreth et al. 2006). Lactic acid bacteria have been proposed to influence the intestinal motility by lowering the pH in the colon, which in turn enhances peristalsis (Salminen and Salminen 1997); however, attempts to use probiotic products for the treatment of constipation have yielded conflicting results. Chmielewska and Szajewska (2010) systematically reviewed RCTs on the effect of probiotics on constipation. The probiotic strains *E. coli* Nissle 1917, *L. casei* Shirota, and *B. lactis* DN-173 010 were found to effectively relieve constipation among adults, whereas neither *L. rhamnosus* GG nor *L. rhamnosus* Lcr35 were effective in the two pediatric studies (Chmielewska and Szajewska 2010). Recently, *B. lactis* HN019 was shown to significantly decrease the whole gut transit time in adult subjects (Waller et al. 2011). *L. rhamnosus* GG has had no effect on the bowel movement frequency or stool form of constipated adults either (Holma et al. 2010). In a pilot study, however, with children aged 4–16 years old, a probiotic multistrain supplement increased bowel movement frequency and softened stool (Bekkali et al. 2007). In elderly subjects, a case study showed a 24% increase in defecation frequency among the probiotic-consuming subjects, but no changes in laxative use were observed (Ouwehand et al. 2002). Taken together, studies on adult populations have been more successful in relieving constipation than studies with children, but the amount of data is still insufficient.

It may be concluded that the effects of probiotic bacteria on the bowel habits of healthy subjects are rather modest, which can be seen as a positive finding, as it suggests that the probiotic products are well tolerated. When symptoms are encountered, the distinct symptoms are likely to react to probiotic treatment in a similar manner among healthy subjects with occasional bowel symptoms and subjects found to have FBDs.

### 23.4 Inflammatory Bowel Disease

Inflammatory bowel disease (IBD) is a common name given to a group of chronic inflammatory conditions in the GI, including Crohn’s disease (CD), ulcerative colitis (UC), and pouchitis.
Environmental, genetic, and immunological factors, together with the GI microbiota, are associated with IBD, which presents symptoms and responds to treatment in a considerably subject-specific manner (Schirbel and Fiocchi 2010). The role of the GI bacteria in IBD is different from that of conventional pathogens since IBD reacts to anti-inflammatory treatment (Friswell et al. 2010). Rather, the GI microbiota species composition in IBD appears to be in a dysbiotic state as specified by decreased numbers of *Faecalibacterium prausnitzii* in CD, affecting the risk of inflammatory relapses (Sokol et al. 2009; Willing et al. 2010; Walker et al. 2011). Probiotic bacteria have been proposed as therapeutic agents for IBD (Gionchetti et al. 2002), and early clinical studies have suggested that probiotic bacteria, such as *E. coli* Nissle 1917, are effective in the treatment of CD (Guslandi et al. 1997; Malchow 1997). However, the effectiveness of probiotics seems to vary between different IBD subtypes.

In the case of CD, the results on probiotic intervention studies have thus far not been encouraging (Doherty et al. 2009). In a recent meta-analysis on RTCs applying *Lactobacillus* species as probiotics for CD, the supplements given together with the maintenance therapy were either ineffective or increased the risk of relapse, but no significant side effects were observed (Shen et al. 2009). Likewise, Doherty and colleagues (2010) found a probiotic treatment to be ineffective in the prevention of postoperative relapse in CD in comparison with antibiotic treatment (nitroimidazole), but suggested that the use of probiotics after an initial antibiotic course could be beneficial in lengthening the remission.

Probiotic intervention studies on UC have been more encouraging. Especially in the maintenance of remission, rather than induction of remission, intervention studies with a multispecies probiotic supplement VSL3# and bifidobacteria have shown a reduced risk of relapse (Sang et al. 2010). In a RCT conducted by Miele and colleagues (2009), VSL#3 in conjunction with steroid and mesalamine medication significantly enhanced remission and prevented relapse during a 12 months follow-up among pediatric UC patients. Correspondingly, adult UC patients consuming VSL#3 also achieved and maintained remission more efficiently (Sood et al. 2009).

Pouchitis, an inflammatory condition, occurs often after ileal pouch anal anastomosis, that is, the construction of an artificial rectum from the distal end of the ileum after surgical removal of the large bowel. The probiotic mixture VSL#3 has been shown to be effective in maintaining remission of chronic pouchitis (Elahi et al. 2008; Holubar et al. 2010). The protective effect has been suggested to be attributed to increased diversity of intestinal microbiota, in particular anaerobic bacteria (Kuhbacher et al. 2006).

Taken together, the evidence for a beneficial effect of probiotics is most convincing in the case of UC and pouchitis, whereas in CD the results are still scarce. In fact, ileal pouch anal anastomosis is usually made in the case of UC not responding to therapy, which could partially explain why these two forms of IBD both respond to probiotic treatment. However, the number of clinical intervention studies is still limited and the treatment scenario in IBD is complex: besides strain selection and dosage, the administration scheme of the probiotic in conjunction with the essential medical therapy is crucial.

### 23.5 Necrotizing Enterocolitis and Short Bowel Syndrome

Necrotizing enterocolitis (NEC) is the most common abdominal emergency of preterm neonates in neonatal intensive care units with a mortality of 15–30% (Braegger 2010). NEC is thought to be attributed to a disturbed intestinal mucosal barrier and an abnormal intestinal microbiota harbored by most preterm infants, resulting from the reduced exposure to maternal microbiota, the consumption of a sterile diet, and the common use of antibiotics. Impressive results were reported
form an open study in a single intensive care unit in Bogotá, Colombia, in which the administration of probiotic bacteria to neonates during 1 year dramatically reduced the incidence and mortality of NEC in the unit compared with the previous year (Hoyos 1999). Thereafter several RCTs on the prevention of NEC by probiotic therapies have been conducted. A recent meta-analysis of 11 RCTs showing no adverse effects concluded probiotic treatment to reduce the incidence of NEC by 30% with high statistical significance (Deshpande et al. 2010). Bifidobacteria, lactobacilli, the yeast *S. boulardii*, and multispecies probiotic supplements were included in the meta-analysis and 9 out of 10 supplements showed a favorable effect for the treatment group (Deshpande et al. 2010). The observation has lead to the suggestion that probiotics should be standard practice for prevention of NEC (Tarnow-Mordi et al. 2010), although this opinion is not shared by all (van Goudoever et al. 2010). The effectiveness of such a wide selection of probiotic supplements could partially be due to the undeveloped gut microbiota of infants. Recently, a mathematical model has been developed to follow the course of NEC using variables for growth of pathogenic and probiotic bacteria in the intestinal lumen and in blood or tissue, the permeability of the intestinal mucosa, and the activity of innate immune cells (Arciero et al. 2010). The model was proposed for use in planning probiotic intervention studies for NEC.

Another GI impairment encountered by children is the short bowel syndrome (SBS), which may either result from intentional bowel resection or a congenital short bowel. Preliminary data suggest that probiotic therapies may be very useful in the treatment of SBS. In two case studies, dramatic improvements in intestinal function and nutritional status were reported in young patients receiving *L. casei* Shirota (Candy et al. 2001; Kanamori et al. 2001). In a small-scale study with SBS patients with enterocolitis, administration of a synbiotic product with two probiotics (*B. breve* Yakult and *L. casei* Shirota) and one prebiotic (galacto-oligosaccharides) resulted in an improvement in the health and nutritional status of the subjects (Kanamori et al. 2004). Probiotics producing l-lactate have also been suggested to be effective in the treatment of d-lactic acidosis in SBS patients (Uchida et al. 2004). While not all intervention studies have shown a beneficial effect (Sentongo et al. 2008), the treatment of SBS with probiotics appears promising. The safety of the treatments is a critical issue, as SBS patients are susceptible to bacterial translocation and bacteremia.

### 23.6 Colorectal Cancer and Carcinogens

The development of cancer is a slow process linked to genetic, physiological, and an array of environmental factors. In the case of colon cancer, the luminal content forms a constant intimate environment for the gut mucosa and a potential risk factor, which can be affected by diet and probiotics. Indeed, most anticancer studies have focused on colorectal cancer, the second most common cause of mortality from malignant disease in Europe (Rafter 2002), but also bladder cancer, the fourth most common cancer in men, has been studied in the context of probiotics (Hoesl and Altwein 2005). There are several mechanisms by which probiotics may accomplish anticarcinogenic effects (Davis and Milner 2009; Kumar et al. 2010). Probiotics have been shown to regulate the activity of the enzymes responsible for converting dietary procarcinogens into carcinogens (Marteau et al. 1990; de Roos and Katan 2000) and to decrease their activity in the feces of healthy volunteers (Goldin and Gorbach 1984; Ling et al. 1994). Additional proposed mechanisms include binding of dietary carcinogens, immune enhancement, protection of the colonic mucosa, and alteration of the intestinal microbiota composition. In addition, radiotherapy may have detrimental effects on the GI microbiota (Crawford and Gordon 2005) and probiotics
have been suggested as therapeutic agents against radiotherapy-associated diarrhea (Salminen et al. 1988; Fuccio et al. 2009).

Currently published epidemiologic studies have yielded contradictory results, with some studies suggesting a reduced risk of cancer, and other studies suggesting no effect (Wollowski et al. 2001), which may relate to strain differences. A report from the International Agency for Research on Cancer comparing the incidence of colon cancer in Denmark (higher incidence) and Finland (lower incidence) suggested a possible protective effect attributed to the consumption of dairy products and the increased fecal counts of lactobacilli in the low-incidence areas (Maclennan and Jensen 1977). A case–control study from France suggested an inverse relationship with risk of large adenomas and yogurt consumption (Boutron et al. 1996). However, in a more recent human clinical intervention study using biomolecular markers related to cancer as outcome measures, only the GI microbiota was shown to be effected (Worthley et al. 2009).

To conclude, the anticarcinogenic effect of probiotics is well justified by in vitro and animal studies. However, due to the complex and slow manner in which cancer develops, executing RCTs to evaluate the effectiveness of probiotics is practically unachievable. An alternative approach is to evaluate the effect using accepted markers of elevated cancer risk and to use epidemiological data when available.

23.7 *Helicobacter pylori* Eradication

*Helicobacter pylori* infection may result in gastritis predisposing to peptic ulcers and eventually increasing the risk of gastric cancer. Therefore the eradication of *H. pylori* is important. Traditionally this has been achieved with a combination of antibiotics and proton-pump inhibitors, often resulting in several adverse effects during treatment. An increasing number of studies suggest that probiotic treatments improve *H. pylori* eradication rates significantly when used in combination with standard anti-*H. pylori* regimens (Canducci et al. 2000; Sheu et al. 2002; Tursi et al. 2004; Sykora et al. 2005; Sheu et al. 2006), whereas in an Argentinian study no additional benefit with a commercial yogurt containing *Bifidobacterium animalis* and *L. casei* strains was observed (Goldman et al. 2006).

Probiotics have also been shown to be effective in reducing the prevalence and severity of side effects such as diarrhea, pain, and flatulence resulting from *H. pylori* antibiotic treatments (Armuzzi et al. 2001; Cremonini et al. 2002; Myllyluoma et al. 2005; Cindoruk et al. 2007) and in balancing the GI microbiota during the antibiotic treatment (Madden et al. 2005; Plummer et al. 2005; Myllyluoma et al. 2007). The total side effects were, however, not significantly alleviated with lactobacilli or fermented milk-based probiotics according to recent meta-analyses (Sachdeva and Nagpal 2009; Zou et al. 2009).

In conclusion, as recently reviewed by Wolvers and colleagues (2010), probiotic administration together with conventional *H. pylori* treatment has been shown to enhance the eradication rate and to reduce the side effects of the treatment. However, successful eradication of *H. pylori* in humans using administration of probiotic bacteria as the primary regimen seems unlikely.

23.8 Lactose Maldigestion

With the exception of the populations of Northern European origin, lactose maldigestion resulting from lactase deficiency affects the majority of adults worldwide. Lactose maldigesters tolerate yogurt usually better than nonfermented dairy products, due to the lactase activity of the yogurt
starter cultures (Kolars et al. 1984). However, it is important to remember that strain-specific differences in lactase activity may vary 100-fold, even between the strains used normally in yogurt manufacture (Sanders et al. 1996). Not all probiotic strains are able to ferment lactose or alleviate lactose intolerance, and the effect is stronger in fermented products than in nonfermented products. Lactase production is strain dependent, but also depends strongly on the growth conditions of the bacteria. Lactase production is strong when lactose is the sole source of energy, but is reduced significantly in the presence of other energy sources such as glucose (Jiang et al. 1996). This may explain why the yogurt bacteria are able to degrade lactose from the yogurt itself, but fail to have effect on additional lactose consumed together with the meal (Martini et al. 1991).

The ability of several lactic acid bacteria starter strains and probiotic strains to improve lactose digestion has been documented (de Vrese et al. 2001). Kefir, a traditional fermented milk drink containing several species of lactic acid bacteria and yeast, has also been reported to improve lactose digestion (Hertzler and Clancy 2003). In addition to bacterial lactase activity, the mechanisms are believed to involve delayed GI transit, improvement of the functions of the commensal microbiota, and reduced sensitivity to symptoms (de Vrese et al. 2001).

23.9 Conclusions

The well-being of our gut may benefit from supplementation with specific probiotic strains, to several well-conducted clinical studies thus far published. However, as probiotic effects are strain specific, conclusions based on meta-analyses with several kinds of probiotic supplements included should be drawn with consideration. Indeed, for most probiotic supplements there is still a high demand for several independent clinical studies assessing various potential health benefits. In a strain-specific manner, however, probiotics may enhance the recovery of even severely dysbiotic states in the GI microbiota (infectious diarrhea, AAD, CDAD, and NEC), protect from inflammation (UC and pouchitis), and alleviate functional GI symptoms.

References


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Chapter 24

Human Studies on Probiotics: Infants and Children

Hania Szajewska

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24.1 Introduction

Despite many years of extensive research, the role of probiotics in the treatment or prevention of diseases often remains uncertain. The use of probiotics continues to rely on health claims made by the manufacturers. Until health and nutritional claims regulate the use of probiotics, it is crucial that
Clinicians understand the various strains and preparations that are commercially available and are able to advise use of these products accordingly. This chapter describes literature pertaining to the clinical effects of the use of probiotics in the pediatric population. Data were identified by searches of the Cochrane Database of Systematic Reviews, the Cochrane Controlled Trials Register, and MEDLINE databases (all up until July 2010) as well as references from relevant articles. The search was limited to randomized controlled trials (RCTs) or their systematic reviews/meta-analyses, which were identified using relevant keywords. For conditions that had previously been reviewed in a systematic review/meta-analysis, only the findings of those reviews are summarized. In the event that relevant primary RCTs were published after the systematic review/meta-analysis was published, these were also included and reviewed. The MEDLINE database was also searched for published, evidence-based clinical practice guidelines developed by respected scientific societies or expert groups. Of note, the effect of probiotics is strain specific. Caution should be exercised so one does not overinterpret the results when all probiotics have been evaluated together.

### 24.2 Probiotics in Infant Formulae

There are four major issues related to the addition of probiotic bacteria to infant formulae. First, timing; that is, the administration often begins in early infancy, sometimes at birth, when infant formulae may represent the only source of feeding in non-breast-fed infants. Second, duration; that is, the daily administration of such products is often prolonged (several weeks or months). Third, the onset of administration is at the time when gut microbiota is not fully established. Finally, delivery is in the form of a specific matrix (infant formula) that could be the infant’s only source of feeding.

In 2010, the Committee on Nutrition of the European Society for Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) systematically reviewed published evidence related to the safety and health effects of the administration of formulae supplemented with probiotics compared with unsupplemented formulae (ESPGHAN Committee on Nutrition 2011). The Committee concluded that for healthy infants, available scientific data suggest that the administration of probiotic-supplemented formula to infants is safe with regard to growth and adverse effects. The administration of probiotic-supplemented infant formula during early life (≤4 months of age) does not result in any consistent clinical effects. The administration of a few probiotics (single or in combination) added to infant or follow-on formulae and given beyond early infancy may be associated with some clinical benefits, such as a reduction in the risk of nonspecific gastrointestinal infections, a reduced risk of antibiotic use, and a lower frequency of colic and/or irritability. However, the available studies varied in methodological quality, the specific probiotics studied, the durations of the interventions, and the doses used. The Committee considers there is still too much uncertainty to draw reliable conclusions from the available data. The safety and clinical effects of one probiotic microorganism should not be extrapolated to other probiotic microorganism(s). In general, there is a lack of data on the long-term effects of the administration of formula supplemented with probiotics. Such data would be of particular importance if the effects persist after the administration of the probiotic(s) has ceased.

### 24.3 Treatment of Acute Gastroenteritis

Acute gastroenteritis (AGE) is one of the most common diseases among children. Generally this is a self-limited illness lasting 5–7 days, and thus, the main aim of treatment is to prevent
dehydration, metabolic acidosis, and electrolyte disturbances. In the vast majority of cases of AGE with mild or moderate dehydration, this can be achieved with oral rehydration solutions. Despite the proven efficacy of oral rehydration, it remains underused (Guarino et al. 2001). The main reason for this is that an oral rehydration solution neither reduces the frequency of bowel movements and fluid loss nor shortens the duration of illness, which decreases its acceptance and prompts interest in adjunctive treatments.

Evidence from several meta-analyses of RCTs (Szajewska and Mrukowicz 2001; Van Niel et al. 2002; Huang et al. 2002; Allen et al. 2003) has consistently shown a statistically significant effect and moderate clinical benefit of some probiotic strains in the treatment of acute watery diarrhea, mainly rotaviral, in infants and young children mainly. Only two meta-analyses focused exclusively on the efficacy of only single probiotic microorganisms and found beneficial effects of Lactobacillus GG (Szajewska et al. 2007) and Saccharomyces boulardii (Szajewska and Skórka 2009). Overall, the beneficial effects of probiotics in the management of acute infectious diarrhea seem to be (1) moderate; (2) strain dependent; (3) dose dependent (greater for doses >10^{10}–10^{11} colony-forming units [CFU]); (4) significant in cases of watery diarrhea and viral gastroenteritis but not in cases of invasive bacterial diarrhea; (5) more evident when treatment with probiotics is initiated early in the course of the disease; and (6) more evident in patients living in developed countries.

Given the available evidence, the ESPGHAN and the European Society of Paediatric Infectious Diseases (ESPID) Expert Working Group (Guarino et al. 2008) recently stated that selected probiotics with proven clinical efficacy (e.g., Lactobacillus GG, S. boulardii) that are administered in appropriate dosages, according to the strain and the patient population, may be used as an adjunct to rehydration therapy for the management of AGE in children. Other probiotic strains may also be used provided their efficacy is documented in high-quality RCTs.

Another recent set of recommendations comes from the National Institute for Health and Clinical Excellence (NICE) (National Collaborating Centre for Women’s and Children’s Health 2009). Similar to the ESPGHAN/ESPID guidelines, this set of guidelines is based on systematic reviews of the best available evidence. The NICE guidelines concluded that there is evidence from a high-quality systematic review suggesting that probiotic treatment had a beneficial effect in regard to shortening of the duration of the diarrhea and reducing stool frequency. However, in contrast to the ESPGHAN/ESPID recommendations, the position of NICE is that despite some evidence of a possible clinical benefit, currently it is not appropriate to recommend the use of probiotics. It is noteworthy that the NICE guidelines are addressed to health-care professionals in the United Kingdom where licensed probiotic preparations are not available in the National Health Service.

In summary, selected probiotic microorganisms may reduce the duration and intensity of symptoms of AGE. The use of probiotics with proven efficacy and in appropriate doses as an adjunct to rehydration therapy is recommended by some scientific societies.

24.4 Prevention of Nosocomial Diarrhea

Nosocomial diarrhea is any diarrhea that a patient contracts in a health-care institution. In children, it is commonly caused by enteric pathogens, especially rotavirus (Matson and Estes 1990). Depending on the population, type of hospital, and standard of care, the reported incidence ranges from 4.5 (Ford-Jones et al. 1990) to 22.6 (Ponce et al. 1995) episodes per 100 admissions. Nosocomial diarrhea may prolong the hospital stay and increase medical costs.
Three placebo-controlled RCTs conducted in hospitalized children have evaluated the effectiveness of *Lactobacillus* GG in the prevention of nosocomial gastrointestinal illnesses (Szajewska et al. 2001; Mastretta et al. 2002; Hojsak et al. 2010). One double-blind RCT carried out in Poland that involved 81 children aged 1–36 months revealed that $6 \times 10^9$ CFU of *Lactobacillus* GG administered orally twice daily significantly reduced the risk of nosocomial diarrhea compared with the placebo (relative risk [RR] 0.2, 95% confidence interval [CI] 0.06–0.6, $P = .002$) (Szajewska et al. 2001). Similarly, a study carried out in Croatia in 742 hospitalized children revealed that administration of *Lactobacillus* GG compared with placebo significantly reduced the risk of gastrointestinal infections (RR 0.4, 95% CI 0.25–0.7) (Hojsak et al. 2010). The only RCT that did not document a beneficial effect of *Lactobacillus* GG administration involved 220 Italian children aged 1–18 months. Whereas breast-feeding was effective in preventing nosocomial rotavirus infections, oral administration of $10^{10}$ CFU of *Lactobacillus* GG once daily compared with placebo did not prevent the development of nosocomial rotavirus infections (RR 0.8, 95% CI 0.55–1.3) (Mastretta et al. 2002).

Two double-blind RCTs evaluated the effectiveness of *Bifidobacterium bifidum* (recently renamed *B. lactis*) and *Streptococcus thermophilus* in the prevention of nosocomial diarrhea. The first trial included infants aged 5–24 months ($n = 55$) who were admitted to a chronic care hospital (relatively long stay). Administration of standard infant formula supplemented with *B. bifidum* and *Str. thermophilus* reduced the prevalence of nosocomial diarrhea compared with administration of the placebo (RR 0.2, 95% CI 0.06–0.8) (Saavedra et al. 1994). The second RCT was conducted in 90 healthy infants younger than 8 months, who were living in residential nurseries or foster care centers. In this study, milk formula supplemented with viable *B. lactis* strain Bb 12 did not reduce the prevalence of diarrhea compared with administration of the placebo (RR 0.7, 95% CI 0.4–1.3) (Chouraqui et al. 2004).

In summary, there is currently evidence to recommend the use of *Lactobacillus* GG, but not enough evidence to recommend the use of *B. lactis* and *Str. thermophilus*, to prevent nosocomial diarrhea. No published cost-effectiveness analyses were identified. However, this is a field of potential great benefit.

### 24.5 Prevention of Community-Acquired Diarrhea

Over the past several years, enormous efforts have been made in the development of safe and effective vaccines against enteric infections, mainly rotavirus. The availability of two efficacious and safe rotavirus vaccines with high efficacy against severe rotavirus gastroenteritis, combined with consistent recommendations to include these vaccines in national immunization programs (Vesikari et al. 2008; Committee on Infectious Diseases 2009; World Health Organization 2009), offers promise in reducing the burden of disease caused by this virus. The effects of these vaccines are very encouraging. However, in some circumstances, effective alternative interventions may be considered. For example, use of probiotics may be considered due to their immediate onset of action, activity against microorganisms other than rotavirus, and lower costs.

With regard to *Lactobacillus* GG, one RCT that involved 204 undernourished infants living in a community with a high burden of diarrheal diseases (Peru) revealed fewer episodes of diarrhea in children who received *Lactobacillus* GG compared with a placebo (5.21 vs. 6.02 episodes/child/year; $P = .03$). This benefit was particularly evident in non-breast-fed children aged 18–29 months (4.69 vs. 5.86 episodes/child/year; $P = .005$) (Oberhelman et al. 1999). In contrast, two double-blind, placebo-controlled RCTs carried out in Europe in children attending day care centers did
not demonstrate an effect of *Lactobacillus* GG administration on the prevention of gastrointestinal infections (Hatakka et al. 2001; Hojsak et al. 2010).

Compared with placebo, use of a formula containing *B. breve* C50 and *Str. thermophilus* 065 in healthy infants did not reduce the incidence or duration of diarrheal episodes, but the episodes were less severe (Thibault et al. 2004).

One double-blind, placebo-controlled RCT that compared *L. reuteri* or *B. lactis* treatment with a placebo for the prevention of infections in 201 infants attending child care centers demonstrated a reduction in the number and duration of diarrheal episodes in the infants treated with the probiotics (Weizman et al. 2005).

In summary, several studies have examined the role of probiotics in the prevention of gastrointestinal infections in healthy individuals, particularly those in children's day care centers. Although RCTs have shown a modest effect for some probiotics, the results are not uniform. Confirmatory RCTs are needed.

### 24.6 Prevention of Antibiotic-Associated Diarrhea

A common side effect of antibiotic treatment is antibiotic-associated diarrhea (AAD), defined as otherwise unexplained diarrhea that occurs in association with the administration of antibiotics (Bartlett 2002). AAD occurs in approximately 5–40% of patients between the initiation of antibiotic therapy and up to 2 months after cessation of treatment (Turck et al. 2003; Elstner et al. 1983; Barbut and Maynard 2002). This wide range in incidence reflects the definition of diarrhea used, patient ages, host factors, and the inciting antimicrobial agents. Almost all antibiotics, particularly those active against anaerobes, can cause diarrhea; however, the risk of AAD seems to be higher with aminopenicillins, the combination of aminopenicillins and clavulanate, cephalosporins, and clindamycin (Barbut et al. 1997; McFarland et al. 1990). Although no infectious agent is found in most cases, the bacterial agent commonly associated with AAD, particularly in the most severe episodes (pseudomembranous colitis), is *Clostridium difficile* (Bartlett 2002).

With regard to the use of probiotics for the prevention of AAD, three relevant systematic reviews of RCTs involving only children were found (Szajewska et al. 2006; Johnston et al. 2006; Johnston et al. 2007). The first review (search date: December 2005) identified 6 RCTs involving 766 children. This review (Szajewska et al. 2006) found that treatment with probiotics compared with placebo reduced the risk of AAD from 28.5% to 11.9% (RR 0.4, 95% CI 0.25–0.8). Preplanned subgroup analysis showed that the reduction in the risk of AAD was associated with the use of *Lactobacillus* GG (two RCTs, *n* = 307, RR 0.3, 95% CI 0.15–0.6), *S. boulardii* (one RCT, 246 participants, RR 0.2, 95% CI 0.07–0.6), or *B. lactis* and *Str. thermophilus* (one RCT, 157 participants, RR 0.5, 95% CI 0.3–0.95).

Another systematic review (Johnston et al. 2006) (search date: August 2006) identified 10 RCTs (six of them were included in the above-mentioned meta-analysis) involving 1986 participants that compared the effects of treatment with either *Lactobacilli* spp., *Bifidobacterium* spp., *Streptococcus* spp., or *S. boulardii*, alone or in combination, with various active/nonactive controls (placebo, conventional care, or no treatment) in children up to 18 years of age being treated with antibiotics. The objective of this review was to assess the efficacy of using these probiotics for the prevention of AAD in children. Six studies used a single-strain probiotic agent, and four combined two probiotic strains. The per-protocol analysis for nine trials reporting on the incidence of diarrhea showed statistically significant results favoring treatment with probiotics over active/nonactive controls (RR 0.49, 95% CI 0.32–0.74). In contrast, intention-to-treat analysis showed
nonsignificant results overall (RR 0.90, 95% CI 0.50–1.63). However, as indicted by the authors of this review, the validity of the intention-to-treat analysis in this review can be questioned due to the high losses to follow-up. A third meta-analysis (Johnston et al. 2007) (search date: January 2005) covers data included in the Cochrane Review by the same authors and, therefore, is not discussed here. As with almost all meta-analyses/systematic reviews, these meta-analyses/systematic reviews are limited by the quantity and quality of the existing data. The methodology of the included studies differed and often was suboptimal. Potential limitations included unclear or inadequate allocation concealment and no intention-to-treat analysis. Study limitations also included a small sample size in some trials and no widely agreed on definition of diarrhea.

Subsequent to the publication of the meta-analyses, one recent, double-blind, randomized, placebo-controlled trial evaluated the effectiveness of kefir, a fermented milk similar to yogurt but containing different fermentation microbes, in preventing AAD. This study conducted in 125 children aged 1–5 years revealed no significant difference in the rates of diarrhea during the 14-day follow-up period in children receiving antibiotics in the kefir group compared with the placebo group (RR 0.82, 95% CI 0.5–1.4) (Merenstein et al. 2009).

In summary, administration of probiotics selected on the basis of their effectiveness in children being treated with antibiotics could be useful in reducing the risk of AAD.

### 24.7 Necrotizing Enterocolitis

Necrotizing enterocolitis (NEC) is one of the most serious, life-threatening gastrointestinal diseases, and it is characterized by various degrees of mucosal or transmural necrosis of the intestine. The highest incidence is reported in infants with birth weights below 1000 g, and the incidence decreases with increasing birth weights (Llanos et al. 2002). The exact cause of NEC remains unclear. However, in addition to prematurity, factors such as formula feeding rather than breastfeeding, intestinal hypoxia–ischemia, and colonization with pathogenic microbiota are considered to play a role in the pathogenesis of NEC (Hsueh et al. 2003). It also has been suggested that the enteral administration of probiotics to preterm newborns could prevent infections, prevent NEC, and reduce the use of antibiotics (Caplan and Jilling 2000).

A number of systematic reviews, with or without a meta-analysis, have reviewed data on the effects of the enteral administration of probiotics on the risk of NEC and mortality in preterm infants (Deshpande et al. 2007; Alfaleh and Bassler 2008; Barclay et al. 2007; Deshpande et al. 2010). Among them, the most recent is the updated meta-analysis by Deshpande et al. (2010). This meta-analysis (search date: March 2009) identified 11 RCTs (n = 2176), including four recent trials, and involved 2176 preterm infants. The authors concluded that there are significant benefits of probiotic supplements in reducing the risk of NEC (RR 0.35, 95% CI 0.23–0.55) and all-cause mortality (RR 0.42, 95% CI 0.29–0.62) in preterm neonates. From a methodological point of view, the meta-analysis is very well done. However, caution must be exercised in overinterpreting the results. The major concern with regard to this meta-analysis, as with many other meta-analyses in the area of probiotics, is whether it is appropriate to pool data on different microorganisms. The strong statement in favor of the routine use of probiotics for preventing NEC without clearly stating which probiotic(s), at what dose, for how long, and other considerations, may be considered by some as beyond the evidence. It is also in contrast to the recent recommendation made by the ESPGHAN Committee on Nutrition (2010). The Committee concluded that the presently available data do not permit recommending the routine use of prebiotics or probiotics as food supplements in preterm infants; the Committee also recommended that each probiotic strain and
potential combinations need to be characterized separately for each product. The ESPGHAN Committee on Nutrition considers it premature to support the routine use of probiotics to prevent NEC. However, it also stated that in settings in which the incidence of NEC is high, one may consider the use of probiotics—single or in combination—that are the best studied, with the highest effect size, and the best safety profile (van Goudoever et al. 2010).

In summary, certain probiotics prevent NEC. Whether probiotic supplementation should become the standard of care is still under discussion. Before the routine use of probiotics in preterm infants, data regarding which products should be administered, at what dosages, and for how long are needed.

24.8 *Helicobacter pylori* Infection

Triple therapy using a proton pump inhibitor with clarithromycin and amoxicillin or metronidazole, given twice daily, remains the recommended first choice treatment for *Helicobacter pylori* infection (Gold et al. 2000; Bourke et al. 2005). One major problem with this therapy is the increased resistance to antibiotics used in the triple therapy (Koletzko et al. 2006; Oderda et al. 2007). In addition, adverse effects are commonly experienced by patients receiving *H. pylori* eradication therapy and are reported by about 5–30% of patients receiving triple therapy (Tong et al. 2007). The commonest side effect is gastrointestinal upset manifested by diarrhea, nausea, or vomiting (Huang et al. 2002). Measures to overcome these problems include the use of probiotics, which are live microbial food ingredients that are beneficial to health. The rationale for the use of probiotics as adjunctive treatment for *H. pylori* infection is based on the results of studies that have shown that various lactobacilli (e.g., *Lactobacillus johnsonii* La1, *L. acidophilus* CRL 639, *L. casei*), or their metabolic products, can inhibit or kill *H. pylori in vitro* (Bhatia et al. 1989; Bernet et al. 1994).

In the pediatric population, there have been four RCTs that evaluated whether consumption of probiotics increases *H. pylori* eradication rates and reduces the side effects of treatment. One RCT (Sykora et al. 2005) demonstrated that patients in the probiotic-supplemented group who received omeprazole, amoxicillin, and clarithromycin plus *L. casei* DN-114 001 had greater eradication rates than subjects in the control group who received the drug treatment only. The incidence of side effects, however, did not differ between groups. Three other RCTs revealed no significant differences in the eradication rates between those treated with a commercial yogurt containing *Bifidobacterium animalis* and *L. casei* (Goldman et al. 2006), *L. reuteri* ATCC 55730 (Lionetti et al. 2006), or *Lactobacillus GG* (Szajewska et al. 2009) and those treated with a control product. Some probiotics, however, may reduce the incidence of adverse effects associated with *H. pylori* eradication regimens.

In summary, in children with *H. pylori* infection, supplementation of standard triple therapy with selected probiotics may alter the eradication rate and/or side effects; however, a lack of repeat studies does not allow for definitive conclusions.

24.9 Inflammatory Bowel Disease

The etiology of inflammatory bowel disease (IBD), which consists mainly of two distinct disorders (Crohn’s disease and ulcerative colitis), remains elusive. However, there is increasing evidence that gut microbiota play a role in the pathogenesis of IBD by both initiating and maintaining
inflammation (Ogura et al. 2001; Hisamatsu et al. 2003). Recently, the IBD Working Group of the British Society of Paediatric Gastroenterology, Hepatology, and Nutrition concluded that despite the widespread use of probiotics by pediatric gastroenterologists and families, there is little evidence of their beneficial effect (Sandhu et al. 2010; Wilson et al. 2010).

24.10 Functional Gastrointestinal Disorders

Functional gastrointestinal disorders (FGDs) are defined as a variable combination of chronic or recurrent gastrointestinal symptoms not explained by structural or biochemical abnormalities. In 1999, the Rome II diagnostic criteria for FGDs were formulated, primarily to assist research (Drossman 1999). In 2006, they were replaced by updated Rome III criteria, which were separate for infants and toddlers (Hyman et al. 2006), for children and adolescents (Rasquin et al. 2006), and for adults (Drossman 2006). FGDs account for a substantial number of referrals to gastroenterology clinics. Management remains difficult, prompting interest in new and safe treatment options.

24.10.1 Infantile Colic

This condition is usually self-limited, with no long-term adverse effects; however, it may be very distressing to parents. The therapeutic options for this common problem remain limited. Recently, it has been suggested that probiotics may offer some benefit. One open RCT carried out in 83 breast-fed infants with colic (defined as >3 h of crying on >3 days/week) showed that the administration of *L. reuteri* ATCC 55730 (10⁸ CFU, once daily 30 min after feeding) compared with simethicone (60 mg/day as 15 drops twice a day after feeding) for 28 days resulted in a significant reduction in median crying times. At the end of the study, 39 patients (95%) in the probiotic group were responders (i.e., patients who experienced a decrease in the daily average crying time of 50% during the study) and 3 patients (7%) in the simethicone group were responders (*P* < .001). No adverse effects of *L. reuteri* were reported. Although the mechanism of action of *L. reuteri* for treating infantile colic has yet to be elucidated, these findings are very promising. However, there are some methodological limitations to the study, including no allocation concealment, no blinding, and no intention-to-treat analysis; these limitations may result in selection, performance, and/or attrition biases and, eventually, invalidate the results. Another limitation of the study is the lack of a true placebo group. Thus, there is too much uncertainty to draw reliable conclusions from the available data.

24.10.2 Recurrent Abdominal Pain

A Cochrane systematic review (Huertas-Ceballos et al. 2009) (search date: December 2006) of dietary interventions for recurrent abdominal pain (currently usually classified according to the Rome II criteria) and irritable bowel syndrome (IBS) concluded that there is no evidence that lactobacillus supplementation is effective in the management of children with recurrent abdominal pain.

24.10.3 Irritable Bowel Syndrome

IBS encompasses a group of functional bowel disorders in which abdominal discomfort and pain are often associated with an altered bowel habit and bloating for which there is no evidence of detectable organic disease. In one pediatric study, children with IBS represented 25–50% of visits
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to a gastroenterologist’s clinic (El-Matary et al. 2004). At best, currently available therapies provide symptomatic relief, but there are none that can influence the natural course of the disorder.

Of three double-blind RCTs that evaluated the effects of using probiotics for management of IBS in children, two evaluated the effects of Lactobacillus GG. In the first trial (Bausserman and Michail 2005), no significant difference was found between the group receiving Lactobacillus GG (10^{10} CFU, twice daily, for 6 weeks) and the placebo group in the relief of abdominal pain. The only gastrointestinal symptom that showed improvement with the administration of Lactobacillus GG compared with the placebo was a lower occurrence of perceived abdominal distention \( (P = .02) \). In the second RCT (Gawrońska et al. 2007), children with IBS according to the Rome II criteria who received Lactobacillus GG (3 × 10^9 CFU, twice daily, for 4 weeks) compared with the placebo were more likely to have treatment success (33% vs. 5%). More participants in the Lactobacillus GG group than the placebo group reported reduced frequency of pain \( (P = .02) \) but not reduced pain severity \( (P = .10) \).

The patented probiotic preparation of live, freeze-dried, lactic acid bacteria containing B. breve, B. longum, B. infantis, L. acidophilus, L. plantarum, L. casei, L. bulgaricus, and Str. thermophilus is known as VSL#3. This probiotic preparation was studied in a multicenter, cross-over RCT involving 59 children aged 4–18 years with IBS defined according to the Rome II criteria (Guandalini et al. 2010). In this trial, compared with placebo, VSL#3 resulted in a significant improvement in the subjective assessment of relief of symptoms (the primary outcome) \( (P < .05) \). In addition, there was an improvement in three of four secondary end points, including abdominal pain/discomfort \( (P < .05) \), abdominal bloating/gassiness \( (P < .05) \), and family assessment of life disruption \( (P < .01) \). No significant difference was found between groups \( (P = .06) \) in the stool pattern. No side effect was recorded in any of the patients.

In conclusion, evidence of the effectiveness of probiotics for the treatment of IBS in the pediatric population is very scant. VSL#3 seems to be effective in ameliorating symptoms and improving the quality of life of children affected by IBS. Lactobacillus GG offers some, albeit limited, benefit to patients with IBS.

24.10.4 Functional Constipation

Functional constipation is a frustrating symptom affecting 3% of children worldwide. Treatment is often difficult and long lasting. Moreover, more than 30% of the children dislike the taste of the conventional laxatives available. One rationale for using probiotics to treat constipation is data demonstrating differences in the intestinal microbiota between healthy individuals and patients with chronic constipation (Zoppi et al. 1998; Salminen and Salminen 1997). Second, studies in which B. animalis DN-173 010 was administered have shown improved colonic transit times both in a healthy population (Picard et al. 2005) and in constipated patients (Agrawal et al. 2009). Finally, probiotics lower the pH in the colon, which enhances peristalsis (Salminen and Salminen 1997) and, subsequently, may decrease the colonic transit time.

A recent systematic review of RCTs (search date: May 2009) concluded that there is very limited evidence available from controlled trials to evaluate with certainty the effect of probiotic administration on constipation (Chmielewska and Szajewska 2010). Data published to date suggest that in children, the administration of L. rhamnosus GG (Banaszkiewicz and Szajewska 2005) was not effective in relieving constipation, while the administration of L. casei rhamnosus Lcr35 (Bu et al. 2007) augmented the number of stools and reduced the number of hard stools. Although the results were statistically significant, the overall effects were clinically modest; in addition, the sample size was too small to draw any meaningful conclusion.
Two relevant, additional, double-blind RCTs are now available. One showed that the administration of a fermented dairy product containing \textit{B. lactis} strain DN-173 010 twice a day, for 3 weeks, to 159 children (aged 3–16 years) with constipation (defecation frequency <3 times/week) had no effect on stool frequency or consistency. The rate of success (defined as three or more bowel movements per week and less than one fecal incontinence episode in 2 weeks over the last 2 weeks of product consumption) was higher in the probiotic group compared with the control group (38% vs. 24%, respectively); however, the difference between groups was not statistically significant ($P = .06$). No difference was found in the rate of responders (with a responder defined as a subject who reports a stool frequency $\geq$3 episodes during the last week of product consumption) between the probiotic group and the control group ($P = .31$) (Tabbers et al. 2011).

The next RCT evaluated the effects of \textit{L. reuteri} (DSM 17938) in 44 infants (mean age: 8.2 ± 2.4 months) with functional chronic constipation. Infants in the probiotic group, compared with the placebo group, had a significantly higher frequency of bowel movements at week 2 ($P = .042$), week 4 ($P = .008$), and week 8 ($P = .027$) of supplementation. In the \textit{L. reuteri} group, the stool consistency was reported as hard in 19 infants (86.4%) at baseline, in 11 infants (50%) at week 2, and in 4 infants (18.2%) at weeks 4 and 8. However, there was no significant difference between the \textit{L. reuteri} and placebo groups in stool consistency and crying episodes. No adverse effects were reported (Coccorullo et al. 2010).

In summary, limited available evidence suggests that \textit{L. reuteri} may help infants with constipation, but more studies are needed. Other probiotics studied thus far do not have an effect on functional constipation in children.

### 24.11 Allergy Prevention

The rationale for using probiotics for the prevention of allergic disorders is based on several concepts. First, it has been suggested that improved hygiene and the reduced exposure of the immune system to the microbial stimulus early in childhood contribute to the increasing number of allergic disorders worldwide (Prescot 2003). Second, there are differences in the neonatal gut microflora that may precede or coincide with the early development of atopy. Atopic subjects have more clostridia and tend to have fewer bifidobacteria than nonatopic subjects (Kalliomäki et al. 2001a). Finally, there is evidence suggesting a crucial role for a balanced commensal gut microflora in the maturation of the early immune system.

Two meta-analyses that assessed the effects of probiotics in the prevention of allergic disorders were found (Osborn and Sinn 2007; Lee et al. 2008). In the first meta-analysis (search date: February 2007), 12 studies were eligible for inclusion. Of these, only six RCTs assessed allergic disease and/or food hypersensitivity outcomes. Outcomes were reported for only 1549 of the 2080 infants enrolled in these six RCTs. Although the studies generally had adequate randomization, allocation concealment, and blinding of treatment, many trials had excess losses in patient follow-up (17–61%). Meta-analysis of five RCTs, involving 1477 infants, revealed a significant reduction in infant eczema with probiotic supplementation. However, there was significant heterogeneity among the studies. One study that evaluated the effect of \textit{Lactobacillus GG} use demonstrated that the difference in the prevalence of eczema between the treatment and control groups persisted until 4 years of age (a further RCT—not included in the meta-analysis—showed an effect also at 7 years). However, the findings were no longer significant when the analysis was limited to studies reporting the prevalence of atopic eczema (confirmed by the skin prick test or specific IgE). There were no reports of other benefits for any other allergic disease or food hypersensitivity outcome.
with the administration of probiotics. The authors concluded that there is insufficient evidence to recommend the addition of probiotics to infant feeds for the prevention of allergic disease or food hypersensitivity, as supported by the data reviewed.

The second meta-analysis (Lee et al. 2008) was restricted to trials of probiotics for the prevention and treatment of pediatric atopic dermatitis. PubMed and Cochrane databases (up to July 2007) as well as reference lists were searched. Six prevention RCTs that compared treatment with different *Lactobacillus* species and placebo were included (including five RCTs identified in the previously mentioned meta-analysis). All of the prevention studies were considered to be of high quality. The prenatal and/or postnatal administration of probiotics to pregnant women and their infants resulted in a significant reduction in the incidence of atopic dermatitis in the infants. Exclusion of one RCT involving only postnatal administration of probiotics resulted in a more pronounced effect, suggesting the importance of prenatal administration.

A third review identified three RCTs that evaluated the effect of probiotic use on the prevention of atopic dermatitis. The authors concluded that probiotics, especially *Lactobacillus* GG, seem to be effective for the prevention of this condition (Betsi et al. 2008).

Of note, the effectiveness of *Lactobacillus* GG in the prevention of atopic dermatitis was assessed in one additional RCT (Kopp et al. 2008) not included in the above-mentioned meta-analyses. In this study, which was carried out based on a protocol almost identical to that used in a study by Kalliomäki et al. (2001b), no effect of *Lactobacillus* GG use on the prevention of atopic dermatitis was observed.

In summary, none of the probiotics can be recommended for primary prevention of allergic disorders.

### 24.12 Treatment of Atopic Dermatitis

Atopic eczema is an itchy inflammatory skin condition with associated epidermal barrier dysfunction. Therapeutic options (emollients and topical steroids for mild-to-moderate eczema; topical or systemic calcineurin inhibitors, ultraviolet phototherapy, or systemic azathioprine for moderate-to-severe eczema) are relatively limited and often unsatisfactory in regard to efficacy, thereby prompting interest in alternative treatment methods.

There have been three meta-analyses with conflicting conclusions regarding the role of probiotics in the treatment of eczema. The first meta-analysis (search date: July 2007) assessed the effects of the use of probiotics for the prevention and treatment of pediatric atopic dermatitis (Lee et al. 2008). For the latter outcome, four RCTs (*n* = 299) were identified. The reviewers stated that the clinical significance of the treatment trial findings of the intergroup reduction in the Scoring of Atopic Dermatitis Severity Index (SCORAD) score by −6.64 points (−9.78, −3.49) and −8.56 points (−18.39, 1.28), as well as the intragroup change of −1.06 points (−3.86, 1.73) and −1.37 points (−4.81, 2.07), is questionable. In their view, current evidence is not convincing for a role of probiotics in the treatment of pediatric atopic dermatitis.

A Cochrane Review (search date: April 2008) that identified 12 RCTs involving 781 participants (children only) also concluded that the evidence suggests that probiotics are not an effective treatment for eczema and that probiotic treatment carries a small risk of adverse events (Boyle et al. 2008).

Opposite conclusions were reached by the authors of the third meta-analysis (Michail et al. 2009) (search date: January 2008; 11 RCTs, and data from 10 studies [*n* = 678] available for analysis). Overall, there was a statistically significant difference favoring probiotics compared with
placebo in reducing the SCORAD score. Children with moderately severe disease were more likely to benefit. The outcome was not affected by the duration of probiotic administration, patient age, or type of probiotic used. According to the authors, evidence suggests a modest role for probiotics in the treatment of pediatric atopic dermatitis, with effects observed in patients with moderately severe rather than mild disease.

Finally, Betsi et al. (2008) reviewed 10 RCTs that evaluated probiotics as a treatment for atopic dermatitis. Probiotics were found to reduce the severity of atopic dermatitis in approximately half of the RCTs evaluated.

In summary, the role of probiotics in the treatment of atopic dermatitis remains questionable.

24.13 Respiratory Tract Infections

The successful prevention of respiratory tract infections (RTIs), which are responsible for a significant number of consultations particularly in young children and the elderly, could be useful for patients, families, and society in general. One systematic review (search date: February 2008), without a formal meta-analysis, evaluated the effect of probiotic use for the prevention or amelioration of RTIs. Fourteen RCTs, 12 involving healthy subjects and 2 involving patients with RTIs, were included. In regard to the incidence of RTIs, no significant difference was found between the probiotic and control groups in 10 RCTs, while the use of probiotics was beneficial in the remaining 4 RCTs. In 5 of 6 RCTs that provided relevant data, there was a reduction in the severity of symptoms related to RTIs in the probiotic group compared with the control group. In 3 of 9 RCTs, the clinical course of RTIs was shorter in the probiotic group compared with the control group, whereas no intergroup difference was found in the remaining 6 RCTs. It was concluded that probiotics may have a beneficial effect on the severity and duration of symptoms of RTIs but do not appear to reduce the incidence of RTIs (Vouloumanou et al. 2009).

Subsequent to the publication of the systematic review, one double-blind, placebo-controlled, randomized trial (Leyer et al. 2009) evaluated the effects of the administration of *L. acidophilus* NCFM (10^10 CFU/day), *L. acidophilus* NCFM in combination with *B. animalis* subsp. *lactis* Bi-07 (10^10 CFU/day), or placebo on cold and influenza-like symptom incidence and duration in children. The placebo or probiotics were administered for 6 months (from November through May) to 248 children aged 3–5 years. Compared with the placebo group, the group treated with *L. acidophilus* NCFM had reduced incidences of fever and cough and a reduced antibiotic use. Compared with the placebo group, the group treated with *L. acidophilus* NCFM in combination with *B. animalis* subsp. *lactis* Bi-07 had significantly reduced incidences of fever, cough, rhinorrhea, and any symptom as well as a reduced antibiotic use. Both probiotic groups had significantly reduced symptom duration compared with the placebo group.

Another recent RCT (Hojsak et al. 2010) conducted in 281 Croatian children revealed that compared with the placebo group, children in the *Lactobacillus* GG group had a significantly reduced risk of upper RTIs, a reduced risk of RTIs lasting longer than 3 days, and a significantly lower number of days with respiratory symptoms. There was no risk reduction in regard to lower RTIs. Compared with the placebo group, children in the *Lactobacillus* GG group had no significant reduction in the risk of gastrointestinal infections, vomiting episodes, and diarrheal episodes as well as no reduction in the number of days with gastrointestinal symptoms.

In summary, available data suggest that some probiotics may prevent upper RTIs and also may have some effect on the severity and duration of symptoms of RTIs in children. Repeat studies are needed.
24.14 Conclusions and Future Directions

Probiotics have the potential to prevent and treat many disorders in the pediatric population. However, guidance is needed regarding which microorganism(s) to use for which clinical condition, as well as the timing, dosage, and mode of administration. To date, the best-documented applications are the treatment of AGE, prevention of nosocomial diarrhea, and prevention of AAD. In preterm infants, the most promising application is the prevention of necrotizing enterocolitis, although the routine use of probiotics for this condition remains controversial. For some other indications, there is still too little solid evidence to support their use; further studies investigating the role of particular probiotics in clinical practice are required. As not all probiotics are created equal, the efficacy as well as safety should be established for each probiotic product.

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Chapter 25

Human Studies on Probiotics and Endogenous Lactic Acid Bacteria in the Urogenital Tract

Wayne L. Miller and Gregor Reid

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25.1 Introduction

Lactic acid bacteria (LAB) have long been known to play an important role in the health of the urogenital tract, particularly in reducing the risk of bacterial vaginosis (BV), urinary tract infections (UTI), and vulvovaginal candidiasis (VVC).

Based on culturing of the organisms, it was long believed that *Lactobacillus acidophilus* was the dominant species. However, this perception changed in the 1980s and 1990s, with *Lactobacillus*
crispatus and Lactobacillus jensenii identified by culture as being more common (Antonio et al. 1999). Since then, studies employing molecular techniques have revealed a much more complex microbiota with newly discovered organisms such as Lactobacillus iners being shown to dominate a healthy vagina and Atopobium vaginae in BV subjects (Dumonceaux et al. 2009; Forney et al. 2010; Srinivasan et al. 2010; Yamamoto et al. 2009; Zhou et al. 2010). While these studies have, for the most part, reaffirmed lactobacilli as the dominant microbes in the vagina of the majority of healthy women, there is mounting evidence that in some cases a different microbial pattern can exist in the absence of any disease indicators. In this chapter, we will discuss what is known about the microbiota of the urogenital tract and how LAB, especially lactobacilli, might be used as probiotics for maintenance of health and prevention or treatment of disease.

25.2 Endogenous LAB

25.2.1 Microbiota of the Vagina

The first culture-independent studies of microbiota composition employed PCR amplification of the V2–V3 region of bacterial 16S rRNA genes followed by denaturing gradient gel electrophoresis (Burton and Reid 2002). These revealed the presence of L. iners and A. vaginae, bacteria that are difficult to detect by culture-based methods. L. iners, first isolated in 1999 (Falsen et al. 1999), was found to be present in 42% of premenopausal women (Burton et al. 2003) and A. vaginae was most commonly detected in women with BV (Burton et al. 2004). A later study used a combination of PCR amplification of 16S rDNA with clone analysis, bacterium-specific PCR assay of 16S rDNA, and fluorescence in situ hybridization (Fredricks et al. 2005). More recently, techniques such as analysis of terminal restriction fragment length polymorphisms of 16S rRNA genes (Yamamoto et al. 2009), dideoxy sequencing of cloned chaperonin-60 universal target (cpn60 UT) amplicons (Hill et al. 2005; Schellenberg et al. 2009), and bacterium-specific quantitative PCR (Srinivasan et al. 2010) have been used for metagenomic community profiling (Forney et al. 2010; Hummelen et al. 2010; Zhou et al. 2010). Metagenomic community profiling seeks to deduce bacterial species composition by direct analysis of DNA isolated from a particular environment such as the vagina of healthy women or those with a particular condition such as BV.

The diversity of bacteria in the vagina is illustrated by several hundred species thus far recovered from various cohorts. However, in terms of core members of the microbiota, there are relatively few species, depending on the populations being studied.

A “healthy” vaginal microbiota is not easy to define. While lactobacilli dominate in most healthy women, recent research indicates that outliers exist. In a study by Kim et al. (2009), for example, one of eight “healthy” women (i.e., with no symptoms of disease) had a very diverse microbiota that was not dominated by lactobacilli, but contained significant numbers of Gardnerella vaginalis, an organism usually associated with BV. This could be viewed as meaning a BV-like microbiota may be normal in some women. However, a more likely scenario is that G. vaginalis has different forms, some of which are nonpathogenic (Harwich et al. 2010).

In terms of abundance, Firmicutes (such as Lactobacillus species) tend to dominate in healthy women, followed by Proteobacteria (such as Escherichia coli), Actinobacteria (such as Gardnerella and Bifidobacteria), Fusobacteria, and Bacteriodetes (Kim et al. 2009). For women whose normal microbiota is dominated by lactobacilli, the most abundant species are L. crispatus, L. gasseri, L. iners, and L. jensenii (Kim et al. 2009; Yamamoto et al. 2009; Forney et al. 2010; Hummelen et al. 2010; Zhou et al. 2010). Of these species, L. iners is perhaps the most intriguing. It is a fastidious,
obligate anaerobe that is difficult to culture—so much so, that it was not even detected before the use of culture-independent techniques. Despite this, *L. iners* can be found both in healthy and BV subjects (Tamrakar et al. 2007; Hummelen et al. 2010; Zozaya-Hinchliffe et al. 2010). Exactly how this organism, with the smallest genome of LAB reported to date (Macklaim et al. 2010), is able to persist when other lactobacilli have been eliminated, remains to be determined, as does the extent to which it plays a role in re-establishment of a healthy state naturally or after antimicrobial use.

Over the course of her life cycle, a female’s vaginal microbiota undergoes changes that are linked to levels of hormones particularly estrogen. Estrogen stimulates epithelial cells to produce glycogen, which can subsequently be metabolized to glucose, an energy source that can be used by lactobacilli. The metabolism of glucose by lactobacilli leads to the release of lactic acid, which lowers the pH of the vaginal environment from 5 to 3.8–4.2 and has an antimicrobial effect, particularly on bacteria such as uropathogenic *E. coli* (Valore et al. 2002; Cadieux et al. 2009). Before puberty, estrogen levels are low and the vaginal epithelium is thin and lacking in glycogen. During this time, lactobacilli are low in abundance, and other bacteria, mainly Gram-negative rods and Gram-positive cocci dominate. The increase in estrogen levels after menarche leads to a shift in the microbiota, with lactobacilli becoming more dominant. Even over the course of a menstrual cycle, the microbiota can fluctuate, with increases in the number of lactobacilli during ovulation and a subsequent reduction during menses accompanied by increased risk of BV (Srinivasan et al. 2010; Eschenbach et al. 2000). After menopause, a return to lower levels of estrogen leads to a decline in the abundance of lactobacilli and again increases the risk of BV, an effect than can be reversed with hormone replacement therapy (Heinemann and Reid 2005).

Differences in microbiota composition are also observed between ethnic groups. In a study of North American women, 33% of black women were found to have a microbiota that was not dominated by lactobacilli, compared with 7% of white women (Zhou et al. 2007). In Japanese women, a normal vaginal microbiota that contains non-*Lactobacillus* species at relatively high frequencies is also more common, but not as much as in North American black women (Zhou et al. 2010). Whose microbiota is dominated by lactobacilli, typically only one species of *Lactobacillus* is present in significant numbers whereas Japanese and Caucasian women tend to have multiple species of *Lactobacillus*.

Black women have been reported to be more susceptible to BV (Zhou et al. 2007) and thereby have a greater incidence of preterm birth (Hitti et al. 2007). Differences have also been observed between white and black populations in terms of increased risk of acquiring a sexually transmitted disease associated with BV (Peipert et al. 2008). However, there is nothing to suggest that simply being black is the cause of these differences. Rather, socioeconomic status, hygiene practices, number of sexual partners, and other factors likely explain the higher incidence of an aberrant microbiota in black women.

### 25.2.2 Microbiota of the Urinary Tract

The bladder, ureters and kidneys of both males and females is essentially regarded as sterile, with the distal urethra being colonized by various nonpathogenic bacteria (Liu et al. 2009). It seems likely that bacteria do ascend on a regular basis from the distal to proximal urethra and into the bladder, but they are invariably flushed out by micturition or are unable to survive the conditions. In women who have UTI, the pathogens ascend from the urethra using various adhesins, flagella, and virulence factors (Nielubowicz and Mobley 2010).

In contrast to the plethora of studies on vaginal microbiota composition, few have looked at the bacterial species present in the male urethra, and these have been limited to culture-based,
aerobic studies. There appears to be an abundance of Gram-positive, aerobic bacteria that are unevenly distributed through the anterior urethra. Coagulase-negative *Staphylococcus* species, *Corynebacterium* species, and *Enterococcus* species are the most commonly isolated from the normal male urethra (Montagnini Spaine et al. 2000).

### 25.3 Probiotics for Urogenital Health

Probiotics (defined as live microorganisms, which, when administered in adequate amounts, confer a health benefit on the host) have been studied for the prevention of UTI and VVC, as well as for the prevention and treatment of BV. In addition, probiotics have been used to augment traditional antimicrobial therapy.

#### 25.3.1 Origin and Characteristics of Probiotic and Candidate Probiotic Strains

The concept of administering probiotic lactobacilli for urogenital health emerged from clinical observations in 1973, which showed that women with no history of UTI had an abundance of lactobacilli in the vagina (Bruce et al. 1973). The concept was to replenish the ‘normal’ or ‘good’ bacteria in the vagina and thereby counteract pathogens before they are able to ascend into the bladder (Bruce and Reid 1988). Between 1985 and 2002, candidate probiotic strains were selected on the basis of production of antimicrobial substances, such as bacteriocins and hydrogen peroxide, ability to adhere to epithelial cells, and on *in vitro* inhibitory activity against urogenital pathogens (Reid et al. 1987; Mastromarino et al. 2002; McLean and Rosenstein 2000; Osset et al. 2001). Hydrogen peroxide became of particular interest based on the observation that lactobacilli most frequently isolated from healthy women were capable of producing it, whereas lactobacilli isolated from women with BV were most often anaerobic, non-hydrogen peroxide-producing species (Eschenbach et al. 1989; McGroarty et al. 1992). The role of hydrogen peroxide in probiotic activity has not yet been fully characterized. In a study of 60 vaginal *Lactobacillus* strains, the four with the greatest activity against BV-associated bacteria were all capable of producing hydrogen peroxide, yet it is not clear that this was the sole factor responsible (McLean and Rosenstein 2000). Not only that, but studies have shown that inhibition of BV-associated bacteria can occur without hydrogen peroxide production (Mastromarino et al. 2002; McGroarty and Reid 1988) and while it is toxic to *G. vaginalis* at high concentrations (Klebanoff et al. 1991), it is ineffective against *G. vaginalis* biofilms (Patterson et al. 2007; Saunders et al. 2007).

Selection of strains based on adherence to epithelial cells led to the isolation of highly adherent strains that were expected to persist longer when administered as a probiotic. To date, however, there has been only one study in which *in vitro* adherence has been correlated with increased persistence *in vivo* (Reid et al. 1995).

Today the ability to reach and persist in the vagina and produce metabolites that are beneficial to the host are considered to be more important factors than the *in vitro* ability to adhere to epithelial cells or mucus. In addition, the ability to survive low pH, cope with hormonal changes, modulate host responses, outcompete and displace organisms already present, and, if oral administration is intended, the ability to survive passage through the gastrointestinal tract, are all regarded as important urogenital probiotic attributes (Burton et al. 2003; Reid et al. 2009; Christensen et al. 2002). Mechanisms also appear to include the disruption of pathogen-containing biofilms.
Studies on Probiotics and Endogenous Lactic Acid Bacteria

(Saunders et al. 2007) and interruption of bacteria–bacteria signaling and toxin production (Laughton et al. 2006), inhibition of pathogens by coaggregation (Ekmekci et al. 2009), and production of biosurfactants (Velaars et al. 1998).

As an antimicrobial, lactic acid produced by LAB has inhibitory activity against some pathogens, but it may not have as broad a spectrum of activity as was once thought. There are, in fact, a number of pathogens that are not affected by acidic conditions and *Atopobium* is actually a producer of lactic acid. Hydrogen peroxide is another antimicrobial substance whose role is being re-evaluated. While its antimicrobial activity made it regarded as being critical for health, it is now recognized that hydrogen peroxide–producing strains are readily displaced by pathogens infecting the bladder and vagina. Studies are needed to better understand how these apparently potent protectors of the vagina are seemingly easily displaced by pathogens. Perhaps this is a stepwise process, in which BV pathogens, such as *Gardnerella* and/or *Atopobium* resistant to hydrogen peroxide, propagate and create a micro-environment less suitable for hydrogen peroxide–producing lactobacilli, and ideal for organisms such as *Prevotella* and *Lachnospiraceae*. This might explain why four clusters associated with BV have been identified by deep sequencing (Hummelen et al. 2010). Furthermore, hydrogen peroxide is believed not to be produced by *L. iners*, an organism capable of persisting in the presence of pathogens while other lactobacilli have been displaced (Reid et al. 2009; Macklaim et al. 2010). Bacteriocins are another category of antimicrobials produced by LAB and long reported with potential to play a role in urogenital health (McGroarty and Reid 1988; Wescombe et al. 2009). However, few urogenital bacteriocins have been identified and studied.

One of the most intriguing, albeit complicated aspects of LAB as probiotics is their potential to modulate the host’s immune response. Vaginal epithelial cells produce a range of antimicrobial compounds and are capable of recognizing pathogen-associated molecular patterns through membrane-bound Toll-like receptors (TLRs) (Witkin et al. 2007a). Signaling cascades triggered by activation of TLRs lead to production of pro-inflammatory cytokines and activation of antigen-specific immunity. In this manner, local IgG and IgA antibody production can be initiated in the endocervix or vagina in response to infection. In fact, one hypothesis of the development of BV is that microbe-induced inhibition of TLRs expression or activity can lead to proliferation of atypical vaginal bacteria (Witkin et al. 2007b). Likewise, probiotic LAB have the potential to modulate the host’s immune system through hydrogen peroxide (Voltan et al. 2008), and induction of granulocyte colony-stimulating factor (Martins et al. 2009), defensins, and IL-8 neutrophil recruitment (Kirjavainen et al. 2008) or other mediators.

### 25.3.2 Application of Probiotics to the Urogenital Tract

The most successful and extensively studied probiotics for vaginal health are *L. rhamnosus* GR-1 in combination with *L. reuteri* RC-14 (originally *L. fermentum*). These strains were selected for their ability to inhibit the growth and adhesion of pathogens to epithelial cells (Chan et al. 1984; Reid et al. 1987). Following early clinical studies with *L. rhamnosus* GR-1 on its own and with *L. fermentum* B-54, the *L. reuteri* RC-14 strain was selected because of its greater *in vitro* activity against Gram-positive pathogens, thereby complementing the activity of GR-1 against Gram-negative pathogens. This combination was developed in a dried capsule formulation, and numerous studies have demonstrated their effectiveness in clinical trials.

Both organisms can be detected in the vagina several weeks after instillation (Reid et al. 1994; Cadieux et al. 2002; Morelli et al. 2004), compared with gastrointestinal probiotic, *L. rhamnosus* GG, which was no longer detectable 5 days post administration (Gardiner et al. 2002). The advantage of direct vaginal application is that a large number of organisms may better displace pathogens
and set up an environment conducive to lactobacilli dominance. On the other hand, oral application may help reduce passage of pathogens from the rectum to perineum and vagina (Reid et al. 2003).

25.3.3 Probiotics for BV

BV afflicts many women at any given time, and may be accompanied by symptoms of vaginal discomfort and homogeneous malodorous vaginal discharge. In applying probiotics to subjects with BV, diagnosing the condition is obviously paramount. A number of different methods exist for diagnosis of BV. The Amsel criteria have the longest use and require three out of the following four parameters: homogenous, milky discharge; vaginal pH greater than 4.5; presence of “clue cells” upon microscopic examination of a vaginal smear; and/or positive amine or “Whiff” test (Amsel et al. 1983). Adopted widely since its description in 1991, the Nugent scoring system involves performing a Gram stain on a vaginal smear and enumerating Gram-positive rods presumptive of lactobacilli versus Gram-negative rods and other bacterial morphotypes (Nugent et al. 1991). Other rapid diagnostic systems have been developed based primarily on elevated vaginal pH and sialidase produced by some BV pathogens (Reid et al. 2004; Bradshaw et al. 2005).

A number of clinical studies have demonstrated the potential for probiotics to augment traditional antibiotic therapy, to prevent recurrences and to restore or maintain a normal microbiota (Table 25.1). Due to limited space, few of these will be discussed in depth.

The ability of *L. rhamnosus* GR-1 and *L. reuteri* RC-14 to cure BV following daily intravaginal administration (Anukam et al. 2006b) was an excellent proof-of-concept study, albeit small and not yet repeated. The fact that curing rate was better than vaginal metronidazole was particularly interesting. BV organisms are present in dense biofilms that are recalcitrant to antimicrobial therapy (Swidsinski et al. 2008), and thus while abundance levels may fall, the pathogens are not fully displaced or killed (Hummelen et al. 2010). It is interesting to speculate that the biosurfactants produced by GR-1 and RC-14 might destabilize the BV biofilms, causing displacement and cure of the condition. The potential for probiotic therapy is worth further pursuit, as metronidazole and clindamycin, the main treatment options for the past 40 years or so, are often ineffective, induce drug resistance, and do not prevent recurrences (Bradshaw et al. 2006; Oduyebo et al. 2009). Given the high prevalence of BV, the increase in antibiotic resistance, and lack of advances in managing BV, it is not surprising that women are receptive to probiotics (Anukam et al. 2004).

25.3.4 Probiotics for VVC

In VVC, infection with *Candida* sp. is not due to absence of lactobacilli and cannot be treated with probiotics alone. Rather, antifungal therapy is needed before probiotics can be considered for preventing recurrences. Having stated that, two studies have demonstrated that *L. rhamnosus* GR-1 and *L. reuteri* RC-14, when used in conjunction with traditional antifungal therapy (fluconazole), can improve cure rate and prevent recurrences (Anukam et al. 2009; Martinez et al. 2009b). In addition, *in vitro* cell culture studies have shown that *L. reuteri* RC-14 alone or in combination with *L. rhamnosus* GR-1 can decrease the number of yeast cells recoverable following treatment, as well as increase levels of the antimicrobial cytokine IL-8 (Martinez et al. 2009c; Kirjavainen et al. 2008).

25.3.5 Probiotics for UTI

The prevalence of UTI has not altered since the introduction of antibiotics, and hundreds of millions of women worldwide acquire this debilitating condition each year. Many patients with
<table>
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<tr>
<th>Study</th>
<th>Design (n)</th>
<th>Inclusion Criteria</th>
<th>Intervention</th>
<th>Main Findings</th>
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<tbody>
<tr>
<td>Hemmerling et al. 2009</td>
<td>P1, R, DB, PC (12)</td>
<td>Healthy women</td>
<td>Vaginal applicators containing <em>L. crispatus</em> CTV-05 given at one of three doses (5 × 10⁸, 1 × 10⁹, and 2 × 10⁹ CFU/dose) or placebo once a day for 5 days</td>
<td>Product accepted and well tolerated: occurrence of 45 adverse events, evenly distributed between the probiotic and placebo groups. No subjects (0%) discontinued from the study. 10/12 subjects agreed to statements regarding satisfaction of product.</td>
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<tr>
<td>Antonio et al. 2009</td>
<td>R, DB (90)</td>
<td>Healthy young females aged 14–21 years</td>
<td>Intravaginal capsules containing either 10⁶ or 10⁸ CFU <em>L. crispatus</em> CTV-05 twice daily for 3 days</td>
<td>Moderate colonization efficacy with 60/87 participants colonized. 36/40 subjects colonized who lacked <em>L. crispatus</em>, compared with 24/47 who initially had <em>L. crispatus</em> (p &lt; .001). Decreased colonization associated with protected (p = .02) and unprotected (p &lt; .001) sex.</td>
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<tr>
<td>Reid et al. 2003</td>
<td>R, DB, PC (64)</td>
<td>Healthy women (19–46 years of age), no history of UTI</td>
<td>One capsule containing either <em>L. rhamnosus</em> GR-1 and <em>L. fermentum</em> RC-14 (now called <em>L. reuteri</em>), or placebo, orally, once daily for 60 days</td>
<td>Restoration from asymptomatic BV (Nugent score) to normal microbiota in 37% of lactobacilli-treated group compared with 13% placebo; 6/24 (day 25) and 4/24 (day 56) developed BV in placebo group, none in lactobacilli group</td>
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(continued)
Table 25.1  Studies That Have Examined Use of Probiotics or Candidate Probiotic Strains for Urogenital Health (Continued)

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<tr>
<th>Study</th>
<th>Design (n)</th>
<th>Inclusion Criteria</th>
<th>Intervention</th>
<th>Main Findings</th>
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<tr>
<td><strong>Bacterial Vaginosis (BV)</strong></td>
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<td>Mastromarino et al. 2009</td>
<td>R, DB, PC (34)</td>
<td>BV as diagnosed by Amsel criteria and presence of discharge and/or malodor</td>
<td>One <em>Lactobacillus</em> tablet containing <em>L. brevis</em> CD2 + <em>L. salivarius</em> subsp. <em>Salicinlus</em> FV2 and <em>L. plantarum</em> FV9 (≥10⁹ CFU each) or placebo, administered vaginally once daily for 7 days</td>
<td>Higher cure rate after 7 days in probiotic group (18/18) compared with placebo group (2/16) (p &lt; .001) and after 21 days in probiotic group (11/18) compared with placebo group (3/16) (p &lt; .05)</td>
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<td>Rossi et al. 2010</td>
<td>OL (40)</td>
<td>BV as diagnosed by Amsel criteria</td>
<td>Vaginal tablets containing <em>L. rhamnosus</em> at &gt;10⁶ CFU—one tablet once daily for 6 days, then one tablet twice a week for 2 months, then one tablet once a week until 24 months</td>
<td>Vaginal pH of &lt;4.5 in 4/40 subjects before treatment compared with 24/40 subjects after 12 months and 32/40 subjects after 24 months of treatment (p &lt; .001 and p &lt; .02, respectively), and concurrent reduction of vaginal symptoms (itching, discharge, and burning sensation)</td>
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<td>Martinez et al. 2009a</td>
<td>R, DB, PC (64)</td>
<td>BV as diagnosed by the Amsel criteria and Nugent score (7–10)</td>
<td>Tinidazole (2 g) plus either two oral capsules of <em>L. rhamnosus</em> GR-1 and <em>L. reuteri</em> RC-14 (1 × 10⁹ CFU of each strain) or two placebo capsules taken daily for 28 days</td>
<td>Higher cure rate of BV in probiotic group (28/32) vs. placebo group (16/32) (p &lt; .05)</td>
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<td>Marcone et al. 2008</td>
<td>R, DB (84)</td>
<td>BV as diagnosed by the Amsel criteria</td>
<td>Treated with oral metronidazole 500 mg twice a day for 7 days, or one vaginal tablet of freeze-dried <em>L. rhamnosus</em> once a week at bedtime for 2 months starting 1 week after the last antibiotic administration</td>
<td>Significant long-term improvement (up to 90 days) with the lactobacilli prophylaxis (p = .05)</td>
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<td>Study</td>
<td>Design</td>
<td>Participants</td>
<td>Intervention</td>
<td>Outcomes</td>
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<tr>
<td>Petricevic et al. 2008</td>
<td>R, DB, PC (72)</td>
<td>Postmenopausal women aged 55–65 years with intermediate vaginal microbiota (Nugent score 4–6)</td>
<td>Capsules containing $2.5 \times 10^9$ CFU each of lyophilized <em>L. rhamnosus</em> GR-1 and <em>L. reuteri</em> RC-14 (intervention) or placebo once daily for 14 days</td>
<td>Higher improvement of restoration in probiotic group (21/35) compared with placebo group (6/37) ($p = .0001$)</td>
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<td>Anukam et al. 2006b</td>
<td>R, DB, PC (125)</td>
<td>Premenopausal women with signs and symptoms of BV, positive Nugent, and BVBlue test score</td>
<td>Oral metronidazole (500 mg) twice daily for 7 days plus either capsules containing <em>L. rhamnosus</em> GR-1 and <em>L. reuteri</em> RC-14 or placebo, taken orally twice daily for 30 days</td>
<td>Normal Nugent scores in 43/49 subjects in antibiotic/probiotic-treated group compared with 23/57 in antibiotic/placebo group at 30-day follow-up. Intermediate Nugent score in 6/49 of probiotic group vs. 17/57 of placebo group; and, BV Nugent score in none of probiotic group, and 17/57 of placebo group.</td>
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<td>Marrazzo et al. 2006</td>
<td>R, PC (232)</td>
<td>BV as diagnosed with both Amsel criteria and Nugent Score</td>
<td>Single oral dose of metronidazole (2.0 g) at enrollment and vaginal capsule containing either <em>L. crispatus</em> or placebo, twice daily for three days, then monthly for 3 months</td>
<td>Only 232 of 424 subjects enrolled completed the study, 175/232 were satisfied with capsule as measured by standardized questionnaire, association of satisfaction with improvement of Nugent score. No significant differences between treatment and placebo.</td>
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<tr>
<td>Eriksson et al. 2005</td>
<td>R, DB, PC (187)</td>
<td>BV as diagnosed by Amsel criteria</td>
<td>Clindamycin (100 mg) vaginally, once daily for 3 days; during subsequent menstruation received either lactobacilli-containing tampons (<em>L. gasseri</em>, <em>L. casei</em> var. <em>rhamnosus</em>, and <em>L. fermentum</em>; total $10^8$ CFU per tampon) or placebo tampons</td>
<td>No improvement in cure rate as measured by Amsel criteria (56% probiotic vs. 65% placebo) or Nugent score (55% probiotic vs. 63% placebo) after second menstruation following treatment</td>
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<tr>
<td>Study</td>
<td>Design (n)</td>
<td>Inclusion Criteria</td>
<td>Intervention</td>
<td>Main Findings</td>
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<td><strong>Bacterial Vaginosis (BV)</strong></td>
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<td>Shalev et al. 1996</td>
<td>R, OL (46)</td>
<td>At least four documented episodes of BV or VVC in the past year and no other vaginal pathogens</td>
<td>Crossover trial: group 1—yogurt with <em>L. acidophilus</em> for 2 months, no yogurt 2 months then pasteurized yogurt 2 months; group 2—pasteurized yogurt, no yogurt, then <em>L. acidophilus</em>-containing yogurt, 2 months each</td>
<td>Significant reduction in BV episodes after 1 month (60% to 25% in group 1; 70% to 50% in group 2; <em>p</em> = .004). Significant increase in <em>L. acidophilus</em> in vagina and rectum in group 1 vs. group 2 after 1 or 2 months (<em>p</em> &lt; .05). High participant dropout rate (reasons unknown).</td>
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<tr>
<td>Hallén et al. 1992</td>
<td>R, DB, PC (60)</td>
<td>BV as diagnosed by Amsel criteria</td>
<td>Intravaginal capsules containing either $10^8-9$ CFU <em>L. acidophilus</em> or placebo, twice daily for 6 days</td>
<td>Normal vaginal wet smear results in 16 out of 28 women who were treated with lactobacilli in comparison to none of the 29 women treated with placebo</td>
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<td><strong>Vulvovaginal Candidiasis (VVC)</strong></td>
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<tr>
<td>Anukam et al. 2009</td>
<td>R, DB, PC (59)</td>
<td>Clinical history of VVC and presence of symptoms at enrollment (abnormal, odorless vaginal discharge, dyspareunia, dysuria, localized irritation, or discomfort in vulvovaginal area)</td>
<td>Single oral dose of fluconazole (150 mg) plus one capsule of either <em>L. rhamnosus</em> GR-1 and <em>L. reuteri</em> RC-14 or placebo, once daily for 3 months</td>
<td>At day 7, yeast infection detected in 7/33 subjects in probiotic group vs. 2/14 in placebo; at day 90, 15/19 subjects in probiotic group free of yeast infection vs. 3/7 in placebo group.</td>
</tr>
<tr>
<td>Studies on Probiotics and Endogenous Lactic Acid Bacteria</td>
<td>Martinez et al. 2009b</td>
<td>R, DB, PC (55)</td>
<td>VVC as diagnosed on the basis of vaginal discharge and positive for <em>Candida</em> spp. and at least one symptom (itching and burning vaginal feeling, dyspareunia, and dysuria)</td>
<td>Single dose of fluconazole (150 mg) plus two oral capsules of either <em>L. rhamnosus</em> GR-1 and <em>L. reuteri</em> RC-14 or placebo, once daily for 28 days</td>
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### Urinary Tract Infection (UTI)

| Beereport et al. 2009 (unpublished) | R, DB, PC (252) | Postmenopausal women with recurrent UTIs | 12 months prophylaxis with either TMP/SMX 480 mg QD or oral capsules with 10^9 CFU *L. rhamnosus* GR-1 and *L. reuteri* RC-14 BID | The mean number of UTIs went from 7.0 to 2.8 in the TMP/SMX and 6.8 to 3.1 in the *Lactobacillus* group. |

| Lee et al. 2007 | R, PR (120) | Children with persistent primary vesicoureteral reflux (VUR) after antibiotic prophylaxis for 1 year | Either probiotics (*L. acidophilus* ATCC 4356, 10^8 CFU/g) twice daily or antibiotics (TMP/SMX 2/10 mg/kg) once daily during second year follow-up | No significant difference in incidence of recurrent UTI in probiotics group (11/60) vs. antibiotic group (13/60; *p* = .926); no significant difference in causative organisms between two groups (*p* = .938) |

| Bruce and Reid 1988 | P1 (5) | Females with recurrent UTI | Intravaginal and perineal implantation of *L. casei* GR-1, twice weekly | Infection-free periods experienced in all subjects ranging from 4 weeks to 6 months, with treatment being well tolerated |

**Note:** R, randomized; DB, double-blind; SB, single-blind; PC, placebo-controlled; P1, phase 1 trial; OL, open label; CFU, colony-forming units; PR, prospective study.
a history of UTI recurrences may have an altered immune status toward other infections and be at greater risk for BV; or recurrent BV may make them more susceptible to UTI (Kirjavainen et al. 2009). In preventing UTI with probiotics, the goal is to establish protective microbiota in the vagina to interfere with the ascension of uropathogens to the bladder. Studies of women with recurrent UTI (Bruce and Reid 1988; Reid et al. 1995) showed that intravaginal implantation of lactobacilli resulted in infection-free periods and fewer UTI recurrences.

A commonly used clinical approach to reduce recurrences, especially in children, is to administer daily antibiotics. In a study that compared daily antibiotics with daily lactobacilli prophylaxis in children, the latter therapy proved just as useful as the antibiotics (Lee et al. 2007). A similar outcome has also been reported when *L. rhamnosus* GR-1 and *L. reuteri* RC-14 have been taken orally each day for 1 year in elderly women, with the mean number of reported symptomatic UTIs falling from 7.0 per subject the year before the study to 2.8 with trimethoprim-sulfamethoxazole treatment, and from 6.8 to 3.1 with probiotics (Beereport et al. unpublished).

### 25.4 Future Challenges

There are perhaps three major challenges for the foreseeable future. The first is to understand how and why the vaginal microbiota changes between a healthy and diseased state. If the triggers can be identified, it will make it easier to develop novel interventions through probiotic, prebiotic, and similar means. Such studies might require specific cohorts to be studied, such as those with a high prevalence of BV versus women who have a low frequency of infection. It will also require knowledge of the microbiome and its functionality over time.

The second challenge will be to develop a diagnostic test that detects an aberrant condition associated with complications (increased risk of infection and preterm labor) rather than one that is simply a transient, asymptomatic BV.

The third challenge will be to develop multiple biotic products that allow for some degree of personalized treatment. For example, a woman with cluster A microbiota may need different lactobacilli or a combination of organisms than one with cluster C profiles. Some women may need an intravaginal intervention while others an oral or ointment treatment. The industry is not geared up for such niche products, and it may be that the delay in such approaches being implemented is a financial one rather than technical.

Challenges aside, this is an exciting time for a long-ignored area of women’s health. Hopefully, funding agencies will recognize the critical role that the vaginal microbiota play in human life and reproduction.

### References


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Chapter 26

Lactic Acid Bacteria and Blood Pressure

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26.1 Regulation of Blood Pressure

Increased blood pressure is one of the leading risk factors for cardiovascular diseases and cardiovascular events. According to the general definition of hypertension [systolic blood pressure (SBP) ≥ 140 mm Hg and/or diastolic blood pressure (DBP) ≥ 90 mm Hg], up to 30% of the world’s adult population were hypertensive in 2000 (Kearney et al. 2005). Hypertension often coexists with other risk factors, including hypercholesterolemia, insulin resistance, metabolic syndrome, and arterial stiffness. Altogether these conditions increase cardiovascular morbidity and mortality. Thus, hypertension is an important public health challenge, of which prevention, early identification, and treatment should receive high attention.
Long-term regulation of blood pressure is closely related to kidney function and body fluid volume homeostasis while the short-term control of blood pressure has been attributed to the sympathetic nervous system (Wyss and Carlson 2001). In addition, peripheral resistance and thus arterial tone contribute to the blood pressure regulation. One of the key systems related to kidneys and body fluid volume is the renin–angiotensin system (RAS). RAS consists of a cascade of enzymes and receptors, beginning from renin secreted by kidney juxtaglomerular cells and leading ultimately to the formation of angiotensin II (Ang II) and its binding to Ang II type 1 receptors (AT₁) (Lemarić and Schiffrin 2010) (Figure 26.1). This leads to arteriolar constriction, increase of blood pressure, and water and salt retention via increased production of aldosterone. The importance of RAS in cardiovascular diseases has been demonstrated by the clinical benefits of angiotensin-converting enzyme (ACE) inhibitors and AT₁ receptor blockers. Additionally, in molecular modeling, components of RAS (especially ACE) have been used as potential targets of food-derived antihypertensive compounds (Pripp et al. 2004).

Blood vessels contribute to blood pressure regulation by controlling vascular resistance. Due to aging, increased blood pressure, or other pathophysiological factors, arteries stiffen and
gradually lose their ability to adjust to blood pressure changes (Ghiadoni et al. 2009). In addition, endothelial dysfunction (impaired function of the innermost layer of the arteries) is often observed in the presence of cardiovascular diseases and risk factors, such as hypertension (Taddei et al. 2000).

Although functional food products produced with lactic acid bacteria should not be considered as drugs, they may suit people with high normal blood pressure before pharmacological therapy is required and also thereafter combined with drugs. Functional foods, consumed as a part of the normal diet, may reduce the threshold of commitment to the treatment of hypertension.

26.2 Lactic Acid Bacteria as Producers of Bioactive Peptides

26.2.1 Description of Bioactive Peptides

Milk contains the nutrients that are needed for growth and development and is a rich source of proteins, lipids, lactose, minerals, and vitamins. Besides being an essential requirement for neonates and a traditional food product, milk contains large amounts of physiologically active peptides encrypted in the protein sequences. Bioactive peptides have been defined as specific protein fragments, which have a positive effect on body functions and which may ultimately influence health (Kitts and Weiler 2003). At present, milk proteins are considered the most important source of bioactive peptides (Korhonen 2009). Milk-derived peptides, both from whey and casein fractions, possess a variety of physiological effects. Numerous known peptide sequences exhibit antihypertensive (for review, see Saito 2008), immunomodulatory (Gauthier et al. 2006), osteoprotective and mineral-binding (Möller et al. 2008), antioxidative (Pihlanto 2006), antimicrobial (Tomita et al. 1991; Zucht et al. 1995), antithrombotic (Jollès et al. 1986), or opioid (Antila et al. 1991; Nurminen et al. 2000) activities. Accordingly, milk-derived peptides are potential candidates to be incorporated into food products and used to improve cardiovascular, skeletal, or digestive system function or immune defense or mood.

26.2.2 Formation of Bioactive Peptides

Peptides may be deliberated from their parent proteins by enzymatic hydrolysis during gastrointestinal digestion, fermentation of milk with proteolytic starter cultures, or hydrolysis by enzymes obtained from microorganisms (for review, see Phelan et al. 2009). Peptides may be produced also by chemical synthesis, recombinant DNA technology, or enzymatic synthesis, if the structure of the peptide is known (Gill et al. 1996).

The peptidase systems of lactic acid bacteria utilize milk protein by converting peptides into amino acids, which are further used for protein synthesis, generation of metabolic energy, and recycling of reduced cofactors (for review, see Christensen et al. 1999). Lactic acid bacteria, such as Lactobacillus helveticus and Lactococcus lactis, are traditionally used in dairy food processing. Therefore, in the fermentation process of milk, bioactive peptides can be generated by dairy starter cultures and significant amounts of them may be found from the final product. Tripeptides isoleucine–proline–proline (Ile-Pro-Pro) and valine–proline–proline (Val-Pro-Pro) have been found from sour milk fermented with L. helveticus CP790 and Saccharomyces cerevisiae (Nakamura et al. 1995a). Several cheeses of Swiss origin are also found to contain the same tripeptides (Meyer et al. 2009). The concentration of Ile-Pro-Pro and Val-Pro-Pro seems to increase in the course of ripening process, reaching 100 mg/kg after 4–7 months. However, the stability of peptides in
cheese may be weak. Additionally, the whey fraction of a yogurt-like product fermented by \textit{L. helveticus} CPN4 has been found to contain a dipeptide Tyr-Pro, which produced a significant antihypertensive effect in spontaneously hypertensive rats (SHR) (Yamamoto et al. 1999).

Gastrointestinal enzymes such as pepsin and trypsin may also be utilized to generate bioactive peptides from whole proteins (Pihlanto-Leppälä et al. 2000). Additionally, enzyme combinations can be used. Proteolytic enzymes of lactic acid bacteria, such as the cell wall--associated serine protease, may be isolated, purified, and used to produce bioactive peptides from casein of different species (Minervini et al. 2003). In functional food production, use of commercially available microbial-derived proteinases and ultrafiltration membranes is cost effective and increases product yields (Korhonen and Pihlanto 2003).

### 26.3 Antihypertensive Peptides from Milk Proteins

Both epidemiological and intervention studies suggest that consumption of low-fat dairy products is inversely related to the risk of hypertension (McCarron et al. 1984; Appel et al. 1997; Toledo et al. 2009). These findings have generated further studies on the components of milk possessing antihypertensive effects. Milk is rich in calcium and potassium, of which increased intake has been shown to lower blood pressure (Whelton et al. 1997; van Mierlo et al. 2006). Thus, electrolyte content may partly explain the inverse association between consumption of milk products and blood pressure. In addition to minerals, the antihypertensive effect of milk has been related to milk protein. Most observational studies and clinical trials suggest that increased intake of protein is associated with lower blood pressure and attenuated blood pressure increase over time (Obarzanek et al. 1996; He and Whelton 1999; Burke et al. 2001).

Bovine milk contains about 32 g/l of protein, of which caseins account for 80% and whey proteins constitute the remaining 20% (for review, see Haug et al. 2007). Both casein and whey fractions of milk protein have been shown to decrease blood pressure as such (Pal and Ellis 2009). However, the observed antihypertensive effect can often be attributed to specific peptides encrypted in the parent milk protein. Concerning peptides derived from casein, the most extensively studied are the tripeptides Ile-Pro-Pro and Val-Pro-Pro (see below), but other antihypertensive peptides have been found from casein as well. The hydrolysis of isoelectric casein with pepsin generates peptides corresponding to \(\alpha_{s1}\)-casein f(90–94) (Arg-Tyr-Leu-Gly-Arg), \(\alpha_{s1}\)-casein f(143–149) (Ala-Tyr-Phe-Tyr-Pro-Glu-Leu), and \(\alpha_{s2}\)-casein f(89–95) (Tyr-Gln-Lys-Phe-Pro-Gln-Tyr), which have been shown to exert antihypertensive activity after oral administration to SHR (Del Mar Contreras et al. 2009). These peptides inhibited ACE, the pivotal enzyme in blood pressure regulation, by IC\textsubscript{50} values of 0.7, 6.6, and 20.1 \(\mu\)M, respectively. In addition, \(\beta\)-casein f(133–138) peptide (Leu-His-Leu-Pro-Leu-Pro), identified from milk fermented with \textit{Enterococcus faecalis}, has been shown to produce a significant antihypertensive effect in SHR (Quirós et al. 2007).

\(\alpha\)-Lactorphin (Tyr-Gly-Leu-Phe) and \(\beta\)-lactorphin (Tyr-Leu-Leu-Phe) can be released from the milk whey proteins \(\alpha\)-lactalbumin and \(\beta\)-lactoglobulin, respectively, in enzymatic proteolysis by gastric and pancreatic enzymes (Antila et al. 1991). \(\alpha\)-Lactorphin has been shown to produce a transient, dose-dependent blood pressure–lowering effect, which is abolished by a specific opioid receptor antagonist, naloxyone (Nurminen et al. 2000). Both tetrapeptides improved vascular function of isolated rat mesenteric arteries \textit{in vitro} (Sipola et al. 2002a). Another tetrapeptide from \(\beta\)-lactoglobulin, \(\beta\)-lactosin B (Ala-Leu-Pro-Met), showed strong antihypertensive effect in SHR as well (Murakami et al. 2004). Also proteinase K–digested whey of cheese origin was shown to decrease blood pressure in SHR after single-dose administration (Abubakar et al. 1998). From the
Lactic Acid Bacteria and Blood Pressure

26.3.1 Casein-Derived Tripeptides Ile-Pro-Pro and Val-Pro-Pro

26.3.1.1 Experimental Studies

The effect of tripeptides Ile-Pro-Pro and Val-Pro-Pro and fermented milk products containing them on blood pressure have been investigated both in acute and long-term experimental studies. In these studies, normotensive Wistar-Kyoto (WKY) rats and different models of hypertension, such as SHR and double transgenic rat (dTGR) harboring human renin and angiotensinogen genes have been used. The nonobese, type 2 diabetic Goto-Kakizaki (GK) rat has also been used. In addition, several in vitro and in vivo studies have been performed to obtain more insight into the mechanisms of action and bioavailability of casein-derived tripeptides.

The antihypertensive effect of milk casein–derived peptides was first demonstrated by casein hydrolysate formed by purified proteinase from L. helveticus CP790 and milk fermented with the same bacteria (Yamamoto et al. 1994). Single-dose oral administration of casein hydrolysate or L. helveticus CP790 fermented milk decreased SBP of SHR by 21 or 35 mm Hg after 8 h of administration. An acute blood pressure–lowering effect after oral administration was observed in SHR but not in normotensive WKY rats. It was concluded that the peptides deliberated from casein by extracellular proteinase were responsible for the antihypertensive activity. Thereafter, ACE-inhibitory substances were found to be produced in sour milk during fermentation with L. helveticus and S. cerevisiae (Nakamura et al. 1995b). After isolation, the sequences were identified to be Ile-Pro-Pro-and Val-Pro-Pro, which inhibited ACE by IC50 values of 5 and 9 μM, respectively. This sour milk decreased SBP of SHR by 22 mm Hg after 6 h of oral administration (Nakamura et al. 1995a). Blood pressure of the rats returned to the initial level at 24 h after administration. IC50 values of Ile-Pro-Pro and Val-Pro-Pro can, however, be even lower depending on the concentration of the substrate used in in vitro experiments (Lehtinen et al. 2010). Also a third tripeptide, leucine-proline-proline (Leu-Pro-Pro), has been shown to inhibit ACE (Lehtinen et al. 2010). The amino acid sequences corresponding to Ile-Pro-Pro, Val-Pro-Pro, and Leu-Pro-Pro are found in the primary structure of bovine β-casein (74–76 Ile-Pro-Pro, 84–86 Val-Pro-Pro, 161–163 Leu-Pro-Pro) and κ-casein (108–110 Ile-Pro-Pro) (Farrell et al. 2004) (Figure 26.2).

Long-term studies have been mostly performed using young animals, which still have normal blood pressures (Table 26.1). This has enabled studying the possible attenuating effect of tripeptides on the development of hypertension. The development of hypertension has attenuated significantly in rats receiving either pure Ile-Pro-Pro and Val-Pro-Pro in water or milk products fermented with L. helveticus (and/or S. cerevisiae) containing them (Table 26.1). However, pure tripeptides have not produced as strong antihypertensive effect as the milk products. Minerals (calcium, potassium) present in the milk products have likely contributed to the more pronounced

Figure 26.2 Segment of bovine β-casein amino acid chain.
<table>
<thead>
<tr>
<th>Reference</th>
<th>Duration</th>
<th>Study Characteristics</th>
<th>Dose</th>
<th>Systolic Blood Pressure</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Acute Experiments</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yamamoto et al. 1994</td>
<td></td>
<td>Casein hydrolysate</td>
<td>15 mg/kg peptides</td>
<td>-22 mm Hg after 6 h</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>L. helveticus</em> CP790 fermented milk</td>
<td>15 mg/kg peptides</td>
<td>-35 mm Hg after 8 h</td>
</tr>
<tr>
<td>Nakamura et al. 1995a</td>
<td></td>
<td><em>L. helveticus</em> and <em>S. cerevisae</em> fermented milk</td>
<td>0.3 mg/kg IPP, 0.6 mg/kg VPP</td>
<td>-22 mm Hg after 6 h</td>
</tr>
<tr>
<td><strong>Long-Term Experiments</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nakamura et al. 1996</td>
<td>16 wk</td>
<td>Diet containing 2.5% lyophilized sour milk</td>
<td>Not specified</td>
<td>-19 mm Hg vs. control diet</td>
</tr>
<tr>
<td>Sipola et al. 2001</td>
<td>12 wk</td>
<td>IPP and VPP in water</td>
<td>2.5–3.5 mg/kg/d IPP + VPP</td>
<td>-12 mm Hg vs. control</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>L. helveticus</em> fermented milk</td>
<td>2.5–3.5 mg/kg/d IPP + VPP</td>
<td>-17 mm Hg vs. control</td>
</tr>
<tr>
<td>Sipola et al. 2002b</td>
<td>14 wk</td>
<td><em>L. helveticus</em> fermented milk</td>
<td>0.4 mg/kg/d IPP, 0.6 mg/kg/d VPP</td>
<td>-21 mm Hg vs. control</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>L. helveticus</em> and <em>S. cerevisiae</em> fermented milk</td>
<td>0.2 mg/kg/d IPP, 0.3 mg/kg/d VPP</td>
<td>-10 mm Hg vs. control</td>
</tr>
<tr>
<td>Jauhiainen et al. 2005a</td>
<td>9 wk</td>
<td>IPP and VPP in water</td>
<td>2.0 mg/kg/d IPP + VPP</td>
<td>-8 mm Hg vs. control</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IPP, VPP, and minerals in water</td>
<td>1.7 mg/kg/d IPP + VPP</td>
<td>-13 mm Hg vs. control</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>L. helveticus</em> fermented milk</td>
<td>1.5 mg/kg/d IPP + VPP</td>
<td>-17 mm Hg vs. control</td>
</tr>
<tr>
<td>Study</td>
<td>Duration</td>
<td>Treatment</td>
<td>IPP + VPP (mg/kg/d)</td>
<td>BP Change vs. Control</td>
</tr>
<tr>
<td>-----------------------------------------</td>
<td>----------</td>
<td>---------------------------------------------------------------------------</td>
<td>--------------------</td>
<td>-----------------------</td>
</tr>
<tr>
<td>Jäkälä et al. 2009a</td>
<td>8 wk</td>
<td><em>L. helveticus</em> fermented milk</td>
<td>3.0–4.4</td>
<td>–14 mm Hg vs. control</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Milk product produced by <em>L. helveticus</em> and proline-specific endoprotease</td>
<td>2.9–4.0</td>
<td>–12 mm Hg vs. control</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Milk product produced by <em>L. helveticus</em> and proline-specific endoprotease and containing plant sterols</td>
<td>2.8–4.0</td>
<td>–7 mm Hg vs. control</td>
</tr>
<tr>
<td>Jäkälä et al. 2009b</td>
<td>8 wk</td>
<td><em>L. helveticus</em> fermented milk</td>
<td>5.9–6.6</td>
<td>–11 mm Hg vs. control</td>
</tr>
<tr>
<td></td>
<td>(GK)</td>
<td>Milk product produced by <em>L. helveticus</em> and proline-specific endoprotease</td>
<td>4.6–5.1</td>
<td>–12 mm Hg vs. control</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Milk product produced by <em>L. helveticus</em> and proline-specific endoprotease and containing plant sterols</td>
<td>4.8–5.3</td>
<td>–10 mm Hg vs. control</td>
</tr>
<tr>
<td>Jäkälä et al. 2010</td>
<td>8 wk</td>
<td>Tripeptide powder in water (<em>L. helveticus</em> fermentation)</td>
<td>3.1–4.3</td>
<td>–14 mm Hg vs. control</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tripeptide powder in water (<em>L. helveticus</em> and proline-specific endoprotease)</td>
<td>3.2–4.4</td>
<td>–14 mm Hg vs. control</td>
</tr>
<tr>
<td>Jauhiainen et al. 2010a</td>
<td>3 wk</td>
<td>IPP and VPP in water</td>
<td>10.9</td>
<td>–3 mm Hg vs. control</td>
</tr>
<tr>
<td></td>
<td>(dTGR)</td>
<td><em>L. helveticus</em> fermented milk</td>
<td>5.4</td>
<td>–19 mm Hg vs. control</td>
</tr>
<tr>
<td>Ehlers et al. 2011a</td>
<td>6 wk</td>
<td>Milk product produced by <em>L. helveticus</em> and proline-specific endoprotease and containing plant sterols</td>
<td>3.7–4.4</td>
<td>–16 mm Hg vs. control</td>
</tr>
</tbody>
</table>

*Note:* Spontaneously hypertensive rats were used in all studies unless otherwise indicated. GK, type 2 diabetic Goto-Kakizaki rat; dTGR, double transgenic rat.
antihypertensive effect. In addition, the bioavailability of peptides may be better from milk in comparison to water and improved by other milk components. It seems that the antihypertensive effect of tripeptides is dose related, as shown in the study of Sipola et al. (2002a), in which two fermented milk products containing different amounts of tripeptides were studied. Furthermore, if the treatment either with pure tripeptides or fermented milk product is terminated, the blood pressure of treated rats gradually increases to the same level as with the control rats (Sipola et al. 2001).

In a recent study, older SHR were used to see if a tripeptide- and plant sterol–containing milk product (produced by L. helveticus and proline-specific endoprotease) and a control milk product decreased SBP, which was already high (195 mm Hg) in the beginning of the study (Ehlers et al. 2011a). Six weeks of treatment with the milk product containing tripeptides and plant sterols decreased SBP by 16 mm Hg compared with those rats that received only water. Interestingly, also the control milk product without any active components decreased blood pressure of the rats significantly, although to a lesser extent.

26.3.1.2 Clinical Studies

Antihypertensive effects of milk products fermented with lactic acid bacteria and containing tripeptides Ile-Pro-Pro and Val-Pro-Pro have been studied in a number of clinical studies. Recently, two meta-analyses on antihypertensive peptides derived from different food sources have been performed (Pripp 2008; Xu et al. 2008). Pripp (2008) included 15 clinical trials in the analysis, of which 13 trials concerned milk-derived peptides. Casein-derived tripeptides Ile-Pro-Pro and Val-Pro-Pro were studied in nine of them. Xu et al. (2008) had 12 trials in the analysis and tripeptides were involved in the intervention in all of them.

Significant decreases of 4.8 mm Hg in SBP and 2.2 mm Hg in DBP were found in the meta-analysis of Xu et al. (2008). When a stratified meta-analysis of trials with tripeptides were performed in the study of Pripp (2008), the result was very similar: 4.6 and 2.2 mm Hg in SBP and DBP, respectively. All the trials included in these analyses had lasted at least 4 weeks and involved subjects that had high normal blood pressure (SBP 130–139 mm Hg and/or DBP 85–89 mm Hg) or grade 1 hypertension (SBP 140–159 mm Hg and/or DBP 90–99 mm Hg). The two analyses provide evidence that tripeptides Ile-Pro-Pro and Val-Pro-Pro have antihypertensive effects in prehypertensive and mildly hypertensive subjects after long-term treatment.

In addition, several newer clinical studies report antihypertensive effects by treatment with the products containing tripeptides; SBP decreases of 3 mm Hg (De Leeuw et al. 2009), 4 mm Hg (Germino et al. 2010), and 6 mm Hg (Yoshizawa et al. 2009; Turpeinen et al. 2009) have been observed. However, recent studies by Engberink et al. (2008), van der Zander et al. (2008a), van Mierlo et al. (2009), Usinger et al. (2010), and Jauhiainen et al. (2010b) have not found any significant effect either on SBP or DBP by treatment with tripeptide-containing products.

The effect of tripeptides on blood pressure has been studied mainly in long-term clinical trials. Only a few studies have addressed the acute effects of tripeptides. van der Zander et al. (2008b) showed a significant decrease of 2 mm Hg in SBP over a period of 8 h after ingestion of tripeptide-containing milk product (8.7 mg tripeptides). In the study of Turpeinen et al. (2011), a milk product containing 21.5 mg tripeptides and 2.0 g plant sterols decreased SBP by 2.1 mm Hg and DBP by 1.6 mm Hg within 8 h of ingestion. Other studies have lasted for 4–21 weeks. The hypotensive effect of lactotripeptide-containing products becomes stronger as the intervention is lengthened (Xu et al. 2008). In general, no significant blood pressure–lowering effects have been seen after 2 weeks of intervention, but already after 4 weeks the SBP has decreased significantly.
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from the baseline values. The blood pressure–lowering effect seems greater in patients with higher baseline blood pressure levels. Studies involving a follow-up period show that when the treatment is terminated, blood pressure gradually returns to the baseline within 2–4 weeks (Kajimoto et al. 2001; Seppo et al. 2002; Nakamura et al. 2004).

In contrast to the published data from animal studies in which tripeptides have shown clear antihypertensive effects in different hypertension models, human data are more contradictory. There may be several reasons for this. For example, tripeptides have been given in several product forms in different studies. In most clinical studies, the test products have consisted of sour milk prepared by fermenting skim milk with *L. helveticus* and/or *S. cerevisiae*. As a placebo regular sour milk or artificially acidified milk has been used. In some studies, test products have consisted of powdered fermented milk incorporated into tablets (Aihara et al. 2005; Mizuno et al. 2005) or casein hydrolysate incorporated into capsules (Hirotani et al. 2007). Also fruit juice (Sano et al. 2005) and spread (Turpeinen et al. 2009) has been used as a carrier. Corresponding placebo products have been prepared without the active ingredients. The components of a milk product may influence the peptide absorption, act directly on blood pressure (e.g., minerals), or contain other bioactive peptides in addition to Ile-Pro-Pro and Val-Pro-Pro. Therefore the results from different trials with different products are not directly comparable, especially when the effects on a biological variable are small. Four different kinds of test products containing Ile-Pro-Pro and Val-Pro-Pro have been used in clinical studies: milk fermented with lactic acid bacteria (Mizushima et al. 2004; Jauhiainen et al. 2005b); powdered fermented milk in tablets (Aihara et al. 2005); powdered, enzymatically hydrolyzed tripeptides (Sano et al. 2005; van der Zander et al. 2008a); and milk product produced by *L. helveticus* and proline-specific endoprotease (Turpeinen et al. 2011).

In addition, different bacteria (*L. helveticus*, *S. cerevisiae*, *A. oryzae*) have been used. The dose has varied between the studies; the lowest dose that has been shown to be effective in humans is 2.6 mg of tripeptides per day (Hata et al. 1996), and the highest tested dose is 52.5 mg of tripeptides per day (Jauhiainen et al. 2005b). All these discrepancies may explain the controversial results obtained in the clinical studies. In the analysis of the active components from the final product, attention has often been paid only to Ile-Pro-Pro and Val-Pro-Pro. Therefore, there may be also other components in the test products that have effects on blood pressure.

Although the antihypertensive effect of tripeptide-containing products is less than that of the most effective antihypertensive drugs (e.g., ACE inhibitors, *AT*$_1$ blockers), they provide an option for people with moderately high blood pressure before pharmacological therapy is required. Lifestyle and nutritional factors play a significant role in the prevention and treatment of hypertension and related disorders. Functional foods, consumed as a part of normal diet, may reduce the threshold of commitment to the management of hypertension. Considering this, it is extremely important to investigate lactic acid bacteria and the possibilities they may provide to the development and manufacturing of effective functional food products or food supplements.

### 26.4 Mechanisms of Action

#### 26.4.1 Mechanisms behind the Antihypertensive Effect of Ile-Pro-Pro and Val-Pro-Pro

The first studies by Nakamura et al. (1995b) showed that fermentation of milk with *L. helveticus* produces ACE-inhibitory substances, which were later identified to be Ile-Pro-Pro and Val-Pro-Pro. Ile-Pro-Pro, Val-Pro-Pro, and Leu-Pro-Pro inhibit ACE competitively at the micromolar level,
but do not inhibit ACE2 in physiologically relevant concentrations (Lehtinen et al. 2010). The inhibitory effects of tripeptides on ACE but not on ACE2 have also been shown using porcine ocular tissues (Luhtala et al. 2009).

Ile-Pro-Pro and Val-Pro-Pro were detected from solubilized fraction of abdominal aorta of SHR but not of WKY after the rats had received a single dose of fermented milk containing the tripeptides (Masuda et al. 1996). As ACE activity is significantly higher in aortas of SHR compared with WKY (Nakata et al. 1987; Nakamura et al. 1988), there may have not been sufficient amounts of ACE in WKY aorta to capture tripeptides so that they would remain in the aortic tissue to be detected in the analysis. This would perhaps explain why the antihypertensive effect of tripeptides is seen only in hypertensives. Also long-term treatment with fermented milk products containing Ile-Pro-Pro and Val-Pro-Pro has shown to have effects on the RAS. In SHR, plasma renin activity increased after treatment with tripeptides (Sipola et al. 2002b). In SHR and GK rats, serum ACE decreased after treatment with tripeptides and plant sterols (Jäkälä et al. 2009a, 2009b).

By using the DNA microarray technique, administration of Ile-Pro-Pro and Val-Pro-Pro to SHR for 5 days was shown to increase the expression of endothelial nitric oxide synthase (eNOS) and connexin 40 genes in the aorta (Yamaguchi et al. 2009). These two genes are both related to vasodilatation. In this study, Ile-Pro-Pro and Val-Pro-Pro also slightly increased cyclooxygenase 1 (COX-1) expression and decreased both the nuclear factor kappa B subunit (NF-κB) gene and the peroxisome proliferator activator receptor gamma (PPARγ) gene expression; these genes are related to inflammatory responses. In the study of Ehlers et al. (2011a), 6 weeks’ treatment of SHR with a tripeptide- and plant sterol–containing milk product (produced by *L. helveticus* and proline-specific endoprotease) decreased expression of ACE and interleukin 13 receptor and changed the expression of several signaling pathways related to inflammatory responses (hedgehog signaling pathway, chemokine signaling pathway, and leukocyte transendothelial migration pathway). These studies show that the antihypertensive effect of tripeptides may also involve other pathways and systems besides the RAS.

### 26.4.1.1 Improved Vascular Function

Besides blood pressure, endothelial dysfunction and arterial stiffness are important risk factors for cardiovascular diseases and are often associated with hypertension (for reviews, see Ghiadoni et al. 2009; Tang and Vanhoutte 2010). In experimental studies, the effect of Ile-Pro-Pro and Val-Pro-Pro on arterial function and tone has been studied using isolated rat aortic or mesenteric arterial rings. *In vitro* experiments have been performed to characterize the direct effect of Ile-Pro-Pro and Val-Pro-Pro on endothelium-dependent and -independent relaxation and contraction responses. In the study of Jäkälä et al. (2009c), long-term incubation of isolated mesenteric arteries with the tripeptides resulted in better-preserved endothelial function in comparison to control arteries. Ile-Pro-Pro and Val-Pro-Pro protected endothelial function of SHR mesenteric arteries indicated as more pronounced endothelium-dependent relaxation to acetylcholine. No differences were observed in endothelium-independent relaxation. Sipola et al. (2001) showed with normotensive Wistar rat mesenteric arteries that Ile-Pro-Pro dose-dependently inhibited the Ang I–induced contraction. Inhibition of Ang I–induced contraction was seen also in another study, in which Ile-Pro-Pro and Val-Pro-Pro were administered together on normotensive Sprague–Dawley rat mesenteric arteries (Jauhiainen et al. 2010a). In a recent study by Ehlers et al. (2011b), Ile-Pro-Pro affected the ACE2-Ang-(1–7)-Mas axis of the RAS by potentiating Ang II–, Ang 1–7–, and bradykinin-induced relaxations in SHR mesenteric arteries.
Vascular function studies have also been performed after long-term treatment of the rats with products containing tripeptides Ile-Pro-Pro and Val-Pro-Pro. Jauhiainen et al. (2005a) showed that mesenteric arteries and aortas of rats that had received minerals and pure tripeptides in their drinking fluid for 8 weeks had improved endothelium-dependent relaxation. No differences were observed in endothelium-independent relaxation. A milk product produced by *L. helveticus* and proline-specific endopeptidase containing tripeptides improved endothelium-dependent relaxation of high-salt loaded GK rat mesenteric arteries after 8 weeks treatment (Jäkälä et al. 2009b). The same product also improved mesenteric artery endothelial dysfunction in SHR with established hypertension (Ehlers et al. 2011a). This latter study showed that the vasoprotective effect of tripeptides is mediated by both nitric oxide (NO)- and endothelium-derived hyperpolarizing factor–dependent mechanisms.

A couple of clinical studies have also addressed the effects of casein-derived tripeptides on arterial function and stiffness. Index of endothelial function, reactive hyperemia measured by plethysmography, was significantly increased after 1-week administration of casein hydrolysate containing Ile-Pro-Pro and Val-Pro-Pro to grade 1 hypertensive subjects (Hirota et al. 2007). In contrast, Jauhiainen et al. (2010b) observed no effects on endothelial function, assessed by pulse wave reflection response to sublingual nitroglycerin and salbutamol inhalation, after 12 weeks intervention with a milk product fermented with *L. helveticus* and containing Ile-Pro-Pro and Val-Pro-Pro in grade 1 hypertensive subjects. However, 10 weeks intervention with *L. helveticus* fermented milk product containing Ile-Pro-Pro and Val-Pro-Pro significantly improved the ambulatory arterial stiffness index, which was calculated from the ambulatory blood pressure recordings, in subjects with high normal blood pressure (Jauhiainen et al. 2007a). The same product decreased augmentation index (AIx) significantly in another study, in which grade 1 hypertensive subjects received the treatment for 12 weeks (Jauhiainen et al. 2010b). However, no effects either on AIx or pulse-wave velocity (PWV) were observed after 10 weeks intervention with tripeptide- and plant sterol–containing spread in subjects with high normal blood pressure (Turpeinen et al. 2009). Furthermore, no changes in PWV or AIx were detected during an 8-h period after acute administration of a milk product produced by *L. helveticus* and proline-specific endopeptidase (containing tripeptides and plant sterols) (Turpeinen et al. 2011). In this latter study, however, excretion of cyclic guanosine monophosphate, second messenger of endothelial NO, was significantly higher in the active treatment group than in placebo, suggesting increased NO production and thus a beneficial effect on vascular function.

### 26.4.1.2 Bioavailability

Bioavailability of milk-derived bioactive peptides produced by fermentation with lactic acid bacteria has been questioned. Normally peptides are rapidly metabolized to the constituent amino acids by brush border membrane peptidases after oral administration and the absorption and bioavailability remains very low. However, there are data demonstrating that at least di- and tripeptides may be absorbed intact, enter the circulation, and produce systemic effects (Foltz et al. 2008; Miguel et al. 2008). Bioactive peptides may be absorbed via carrier-mediated transport or paracellular diffusion (for review, see Shimizu 2004). Apparently, tripeptides are actively transported via a specific transporter (PepT1) and oligopeptides via the paracellular route.

Using three different absorption models, Ile-Pro-Pro and Val-Pro-Pro were demonstrated to be transported in small amounts intact across the barrier of the intestinal epithelium (Foltz et al. 2008). The major transport mechanisms of Ile-Pro-Pro and Val-Pro-Pro were shown to be paracellular transport and passive diffusion. Additionally, in humans, Ile-Pro-Pro and Leu-Pro-Pro
were detectable from plasma after ingestion of tripeptide-enriched yogurt (Foltz et al. 2007). Interestingly, measurable plasma Ile-Pro-Pro concentrations were also found after ingestion of a placebo yogurt beverage without added tripeptides. This was suggested to be due to the generation of Ile-Pro-Pro in the intestinal tract from milk proteins by luminal or brush border peptidases. However, in an in vitro study, Ile-Pro-Pro and Val-Pro-Pro were not generated from β-casein by gastrointestinal enzymes, suggesting that the fermentation step is necessary to produce the tripeptides (Ohsawa et al. 2008). In conscious pigs, Ile-Pro-Pro, Val-Pro-Pro, and Leu-Pro-Pro reached the blood circulation intact after intragastric administration (4.0 mg/kg of each tripeptide) with half-lives of absorption and elimination of only a few minutes (van der Pijl et al. 2008). In rats, radiolabeled Ile-Pro-Pro was shown to be absorbed partly intact from the gastrointestinal tract after a single oral dose as well (Jauhiainen et al. 2007b). Radioactivity was found from several tissues, for example, liver, kidney, and aorta. The excretion of Ile-Pro-Pro was slow; even after 48 h there was still some radioactivity left. Ile-Pro-Pro did not bind to albumin or other plasma proteins in vitro. Considering this and the long-lasting retention of the radioactivity in the tissues, accumulation of Ile-Pro-Pro may occur with daily administration in sufficient concentrations to cause blood pressure–lowering effects, for example, by ACE inhibition in the vascular wall.

26.5 Future Aspects

Lactic acid bacteria provide an important and fascinating option to the development of functional food products. In this chapter, antihypertensive effects of several potent milk protein-derived peptides produced by fermentation with lactic acid bacteria have been presented. In addition, vaso-protective effects of tripeptides Ile-Pro-Pro and Val-Pro-Pro have been introduced. Until now, the research has mainly focused on only a few species of lactic acid bacteria; thus, in the future, more research is needed to cover the wide range of highly usable, natural, and safe bacteria to discover the potential they might had in the prevention of cardiovascular diseases. Applications from pharmaceutical technology (e.g., nanoparticles, microencapsulation, lyophilization) may bring new opportunities in the field of nutritional sciences. Multidisciplinary research and open-minded attitude are needed to develop better and more effective functional food products. As the interest on foods possessing health-promoting or disease-preventing properties has been increasing, more emphasis must have been put on the legal regulation of the health claims attached to the products. Manufacturers need to have enough detailed, extensive, and conclusive data on the beneficial effects of their functional food products. However, as the burden of lifestyle diseases is continuously increasing both in developed and developing countries, there is a huge need for functional food products that may be consumed as a part of normal diet to prevent and nonpharmacologically treat conditions such as increased blood pressure and metabolic diseases.

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Lactic Acid Bacteria and Blood Pressure


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Lactic Acid Bacteria: Microbiological and Functional Aspects


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Chapter 27

Probiotics for Companion Animals

Minna Rinkinen

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27.1 Introduction

In farm animal husbandry, probiotics are widely utilized to improve breeding, performance in stressful conditions such as high animal concentration, early weaning, or rapid growth (Fuller 1989). In companion animal medicine, probiotics are also increasingly used to treat and prevent various clinical disturbances, mainly of gastrointestinal origin. The rationale of administering probiotics to companion animals is based on their ability to balance the intestinal microbiota by reducing colonization of pathogens via competitive exclusion and reinforcing host’s immune defense mechanisms particularly in the intestinal mucosa (Anadon et al. 2006).

Probiotic products aimed for pets are gaining more and more popularity in companion animal health care, and there are also probiotic-enriched dog and cat feeds available. As probiotics are regarded as food supplements and not drugs, the quality control of various supplements and pet foods claiming to contain probiotics can be poor. In addition, the scientific evidence to support their beneficial effects is currently quite modest. For admission to the European common market, probiotics intended for animals have to be tested according to the Feed Additive Directive 70/524/EC, both for safety and efficacy (Becquet 2003).

A study evaluated 19 commercial pet foods labeled to contain probiotics. No product contained all of the microorganisms listed on the package label. Five of the products yielded no relevant bacterial growth when cultivated on lactobacilli-selective MRS agar. Eleven of the 19 feed samples contained additional organisms such as *Pediococcus* spp. Both manufacturing and storage can affect the viability of microorganisms in pet food, and it was unclear what the reasons for such marked deviations from the label were (Weese and Arroyo 2003).

27.2 Species Specificity

To be able to exert their beneficial effects, such as immunostimulation, it is believed that probiotics should be species specific, that is, originate from the same animal species that they are fed to, as bacterial adherence to the gut wall is assumed to be unique to each animal species. Ability to adhere to and at least temporarily colonize the host intestinal mucosa is a prerequisite for probiotics to exert their beneficial actions (Saarela et al. 2000). This concept has been challenged later, as probiotic lactic acid bacteria (LAB) have been shown to be able to adhere to the intestinal mucosa of animals of other species than the host it was isolated from (Conway et al. 1987; Rinkinen et al. 2003b; O’Mahony et al. 2009). *Lactobacillus rhamnosus* GG, a widely studied probiotic isolated from a human, has been shown to survive also in the canine gastrointestinal tract (Weese and Anderson 2002).

Most of the probiotics marketed for companion animals are not originally derived from the canine or feline intestinal microbiota. In the European Union (EU) the microorganisms used as feed supplements are mostly LAB belonging to species *Enterococcus* (*E. faecium*) and *Lactobacillus* (e.g., *L. acidophilus*, *L. casei*, *L. farciminis*, *L. plantarum*, *L. rhamnosus*). In addition, certain bacilli (*B. cereus*, *B. licheniformis*, *B. subtilis*), *Pediococcus acidilactici*, and *Streptococcus infantarius* are listed as EU-approved probiotics. Strains of yeast *Saccharomyces cerevisiae* are on the market as well (Anadon et al. 2006).

The source of the probiotic strains in the marketed food supplements is commonly not made known, but it is rather safe to assume that in general they are not of canine or feline origin.
27.3 *E. faecium*—A Problematic Probiotic?

Many commercially available companion animal probiotic products contain enterococci, mainly *E. faecium*, or *E. faecalis* to a lesser extent. Both are natural habitants of the intestine and have beneficial probiotic properties such as good growth and stability, lactic acid production, and ability to adhere to intestinal mucosa (Strompfova et al. 2004).

*E. faecium* is a common pathogen and one of the most notable causes of nosocomial infections in humans (Sava et al. 2010). Enterococci have a notorious ability to rapidly develop and spread antibiotic resistance (Eaton and Gasson 2001). Therefore, the safety and suitability of enterococci as probiotics has been challenged (Franz et al. 1999; Liong 2004).

*E. faecium* may also modify the intestinal microbiota toward a potentially harmful direction. In small animals, *E. faecium* increased both the *in vitro* adhesion and fecal shedding of *Campylobacter* in dogs (Rinkinen et al. 2003a; Vahjen and Männer 2003).

27.3.1 Regulation and Safety of *E. faecium* Probiotics

Multiresistant enterococci have been isolated from dogs and cats, although no correlation to probiotic feeding has been documented (Jackson et al. 2009). Probiotic enterococci could also possess certain safety risks. However, enterococci are widely distributed in nature and are also used in food technology, so not all the strains can be considered as a health risk.

In the EU, living microorganisms are regulated by Council Directive 87/153 on additives in animal nutrition, and authorized enterococci are listed. Strains not listed should not be marketed in EU area. The Council Directive demands that all new enterococcal strains should have a dossier submitted to the authorities where both risks and efficacy are clarified before marketing authorization. All listed *Enterococcus* strains accepted for use as animal feed additives in EU can be considered safe (Becquet 2003). No probiotic enterococcal infections have been reported in veterinary medicine; thus, the risk, according to present knowledge, appears to be limited.

27.4 Modification of Intestinal Microbiota and Competitive Exclusion by Probiotics

Intestinal microbiota is a complex ecosystem consisting of hundreds (>400) of different types of bacteria. Although the knowledge of canine and feline gut microbiota is lesser than that of humans, one can assume similar diversity. Once established, the gut microbiota remains relatively stable throughout life. However, certain probiotic bacteria are documented to modify the intestinal microbiota (Collins and Gibson 1999).

A group of laboratory beagles with permanent jejunal fistulas for intestinal chyme sampling were supplemented for 7 days with a canine-derived LAB cocktail (*L. fermentum* LAB8, *L. salivarius* LAB9, Weissella confusa LAB10, *L. rhamnosus* LAB11, and *L. mucosae* LAB12). Dogs’ jejunal LAB microbiota was studied with denaturing gradient gel electrophoresis method throughout the study period and also after feeding of the LAB mixture had ceased.

Feeding a mixture of those potentially probiotic strains resulted in alterations in the indigenous jejunal LAB microbiota of all five fistulated beagles. During the LAB cocktail supplementation strains, LAB8 and LAB12 dominated the jejunal LAB microbiota. When the supplementation was finished, the fed LAB were soon cleared out and the indigenous LAB reverted, as to be expected.
However, in four dogs, indigenous *L. acidophilus* emerged as the dominant LAB. This particular strain of *L. acidophilus* established itself in the jejunal chyme at high levels, suggesting that supplementation with strains LAB8 and LAB12 resulted in at least temporarily prevailing alteration of the indigenous LAB microbiota. Unfortunately, the jejunal LAB microbiota was followed up only up to 2 weeks so it is not known how long the effect on the indigenous LAB microbiota would have lasted (Manninen et al. 2006).

### 27.4.1 Competitive Exclusion of Potential Pathogens

Term “competitive exclusion” (CE) was first introduced in poultry husbandry to tackle salmonella outbreaks in chicken farming by administering adult chicken intestinal microbiota to newly hatched chicks. CE has turned out to be an effective way to control *Salmonella* spp. colonization in chicken (Stern et al. 2001). It has been suggested that the CE approach could be also extended to other species (Nurmi et al. 1992).

CE of pathogens is thought to be one of the most important beneficial mechanisms of probiotic bacteria (Adlerberth 2000; Rolfe 2000; Reid and Burton 2002). CE by intestinal bacteria is based on bacteria-to-bacteria interaction mediated by competition for available nutrients and mucosal adhesion sites. To gain a competitive advantage, bacteria can also modify their environment to make it less suitable for their competitors. The production of antimicrobial substances, such as lactic and acetic acid, is one example of this kind of environmental modification (Schiffrin and Blum 2002).

To be able to hinder pathogen colonization (and possible subsequent invasion), probiotics are believed to have several pathways. They can reduce the viability of a pathogen by producing noxious substances, such as lactic acid in the case of *L. casei* and *L. acidophilus* against enterohemorrhagic *E. coli* (EHEC) (Ogawa et al. 2001), or nonacidic material (e.g., bacteriocins) like in *L. acidophilus* that was reported to suppress the growth of *Salmonella typhimurium*, EHEC, and *Shigella flexneri* (Coconnier et al. 1997).

Another way to prevent pathogen colonization is to interfere with their adhesion on the mucosal receptors. For example, *L. reuteri* and *L. crispatus* competed with the receptor sites on the host cell with *Salmonella typhimurium* and enterotoxigenic *E. coli* (ETEC) (Todoriki et al. 2001); *L. reuteri* was also effective in preventing the *in vitro* binding of *Helicobacter pylori* to host cell receptors (Mukai et al. 2002).

The effect of probiotic LAB on the CE of pathogens has been demonstrated using human and canine mucosal material *in vitro* (Tuomola et al. 1999; Rinkinen et al. 2003a; Hirano et al. 2003), and *in vivo* in chickens (Hirn et al. 1992) and pigs (Genovese et al. 2000). Probiotic *E. faecium* significantly decreased the canine *in vitro* mucus adhesion and *in vivo* fecal shedding of *C. perfringens* (Rinkinen et al. 2003a; Vahjen and Männer 2003). Hirano and colleagues (2003) showed that the well-adhering stain *L. rhamnosus* was capable of inhibiting the internalization of EHEC to a human intestinal cell line *in vitro*. The result suggests that a close interaction with the host cells may have been responsible for this suppression of EHEC internalization (Hirano et al. 2003). In poultry production, the CE method is proven to effectively reduce the incidence of salmonellae in broiler chicks (Hirn et al. 1992).

### 27.4.2 Effect on Clostridia

Clostridia have been associated with acute and chronic, intermittent enteritis in dogs. The main clostridial organisms associated with gastrointestinal disturbances are enterotoxin-producing *Clostridium perfringens* and *C. difficile* (Weese et al. 2001; Marks and Kather 2003).
27.4.2.1 Clostridium perfringens

*C. perfringens* is an anaerobic, spore-forming bacillus associated with acute and chronic diarrhea in dogs and cats. However, the role of *C. perfringens* as an intestinal pathogen is questionable, as it also commonly resides in the intestinal tract of healthy dogs (Weese et al. 2001; Marks et al. 2002).

*C. perfringens* produces enterotoxins (CPE), which are classified into five toxigenic types (A–E). All *C. perfringens* types can produce CPE, but type A strains are most frequently involved. CPE has been reported to cause nosocomial diarrhea, severe hemorrhagic enteritis, and acute and chronic large bowel diarrhea in dogs (Sasaki et al. 1999). On the other hand, CPE is also found in feces of nondiarrheic animals (Weese et al. 2001), although a significant association was present with diarrhea and detection of CPE (Marks et al. 2002). One study reports *C. perfringens* carrying β2 toxin gene (*cpb2*) isolated from diarrheic dogs, suggesting β2 toxin alone or together with CPE may play a role in canine clostridial diarrhea (Thiede et al. 2001).

Controlling the number of intestinal *C. perfringens* may thus have beneficial consequences especially in dogs prone to chronic or intermittent diarrhea.

27.4.2.2 Clostridium difficile

*C. difficile* is associated with diarrhea in dogs, although it has been frequently isolated also from asymptomatic dogs, thus its role as an intestinal pathogen is debatable (Weese et al. 2001; Marks et al. 2002; Sasaki et al. 1999). *C. difficile*–related diarrhea in humans is principally associated with hospitalization and use of antimicrobials. In dogs, no significant association was found in the prevalence of *C. difficile* along with hospitalization and antibiotic administration, but increased carriage rate was observed in nonhospitalized dogs receiving antibiotics (Marks et al. 2002).

27.4.3 Effects of Selected Probiotics on Intestinal Clostridia

*E. faecium* significantly decreased the canine in vitro mucus adhesion and in vivo fecal shedding of *C. perfringens* On the other hand, in same studies, *E. faecium* increased both the in vitro adhesion and fecal shedding of campylobacters (Rinkinen et al. 2003a; Vahjen and Männer 2003); thus the seemingly positive outcome on fecal *C. perfringens* numbers may lead to potentially risky spreading of a zoonotic pathogen.

*L. acidophilus* DSM13241 enriched dry kibbles were fed to 15 healthy, adult cats for 4.5 weeks. Viability of the added lactobacilli in the cat feed was ensured throughout the study period. Fecal samples were collected in the final 2 weeks, and the effects on fecal flora were evaluated by cultivation (total anaerobes, lactobacilli, and clostridia). Although the authors reported certain technical difficulties in assessment of fecal clostridia, they concluded that *L. acidophilus* DSM13241 supplementation at a daily dose of $2 \times 10^8$ CFU significantly decreased the number of *Clostridium* spp. in the feces of healthy cats (Marshall-Jones et al. 2006).

When dry feed sprayed with *L. acidophilus* DSM13241 was fed to healthy adult dogs, the number of fecal clostridia remained unchanged when evaluated by selective agar plating method. However, the fluorescence in situ hybridization enumeration showed significant reduction in the number and percentage of fecal clostridia in the dogs, reflecting the meagerness of conventional selective culture methods in gastrointestinal microbiology studies (Baillon et al. 2004).

*L. animalis* LA4 isolated from the feces of a healthy adult dog significantly reduced *C. perfringens* counts at 24 h when added to dog fecal cultures in vitro. The in vivo effect of *L. animalis* LA4 on fecal *C. perfringens* shedding was not investigated in the study (Biagi et al. 2007).
Another dog-derived probiotic, *Bifidobacterium animalis* AHC7, was able to modify fecal microbiota in a study involving 11 dogs. The dogs were fed *B. animalis* AHC7 1.5 × 10^9 CFU daily for 6 weeks. Fecal samples were collected before and after probiotic supplementation for evaluation of total aerobes and anaerobes, *Bifidobacterium* spp., *Lactobacillus* spp., *Bacteroides fragilis*, *Clostridium* spp., and *C. difficile*. At weeks 5 and 6, total fecal *Clostridia* counts were significantly reduced. A more detailed assessment revealed that numbers of *C. difficile* were significantly lower at week 6. No effect was seen in the numbers of *C. perfringens*. In addition, the counts of total aerobes, *Bacteroides, E. coli, Lactobacilli*, or *Bifidobacteria* remained the same (O’Mahony et al. 2009).

### 27.5 Chronic and Acute Enteropathies in Dogs and Cats—Do Probiotics Help?

Enteropathies are common complaints in small animal medicine. Probiotics are frequently marketed to alleviate the clinical signs of acute diarrhea and other gastrointestinal disturbances. However, only a limited number of studies have evaluated the effect of probiotic treatment in acute or chronic gastroenteritis.

#### 27.5.1 Acute Gastroenteritis

In a placebo-controlled, randomized, double-blinded study, 15 dogs with acute enteritis, mostly large-bowel diarrhea, or acute gastroenteritis were treated with a probiotic supplement containing *L. acidophilus, P. acidilactici, Bacillus subtilis, B. licheniformis*, and *L. farcimini*. None of the bacteria were of canine origin. The probiotic cocktail significantly reduced the duration of diarrhea but did not have an effect on vomiting. The etiology of the acute enteritis or gastroenteritis was unclear in the dogs included in the study, as is the cause in most cases of acute diarrhea in dogs. No enteric pathogen was deemed a causative agent. It can be speculated whether the faster recovery was gained due to certain changes in intestinal microbiota, or due to positive immunological effects or both (Herstad et al. 2010).

*B. animalis* AHC7, a probiotic strain isolated from canine feces, was evaluated as a treatment in acute diarrhea. Thirty-one adult dogs with an idiopathic acute diarrhea were assigned either to receive probiotic *B. animalis* AHC7 or placebo. The dogs were allowed to be medicated with metronidazole if deemed necessary by the clinician.

*B. animalis* AHC7 fed at 2 × 10^10 CFU daily significantly reduced the time to resolution of diarrhea. The percentage of dogs needing additional metronidazole therapy was smaller in the probiotic group than the placebo group (Kelley et al. 2009). Although *B. animalis* AHC7 is resistant to metronidazole, the medication may have affected the outcome as the diarrhea may have been of etiology susceptible to metronidazole, which is documented to have a beneficial effect in humans having Crohn’s disease, a form of chronic inflammatory bowel disease (IBD) (Sandborn et al. 2007). Thus metronidazole may have had an impact on the dog’s recovery in the study.

### 27.6 Chronic Enteropathies

Common reasons for canine and feline chronic enteropathies are food sensitivity, IBD, or, in dogs, antibiotic responsive diarrheas of undefined etiology (Allenspach and Gaschen 2003; Trepanier 2009).
27.6.1 Probiotic-Induced Effects on Gastrointestinal Tract

The gastrointestinal mucosa forms an important part of body’s immune system. Intestinal microbiota is an important regulator of the gut-associated lymphatic tissue (GALT). A number of studies have shown that probiotic microorganisms contribute to the immune functions via GALT.

Probiotics have shown their beneficial effects on immune functions and inflammatory process in many in vitro tests, animal models, and clinical trials. Especially the actions on gut mucosal immunity and inflammatory process are of interest when treating chronic enteropathies. Probiotics are known to produce immune modulatory effects in humans, and a probiotic combination of three dog-derived lactobacilli (L. acidophilus NCC2628 and L. acidophilus NCC2766) and one L. johnsonii (NCC2767) demonstrated favorable effects on regulatory cytokines in relation to inflammatory cytokines in an ex vivo study (Sauter et al. 2005).

However, when dogs having food-responsive diarrhea were treated with the same probiotic cocktail containing the three lactobacilli, the effects of the probiotic cocktail on dogs’ cytokine mRNA levels were not consistent, and no significant anti-inflammatory or immunomodulatory effect could be found (Sauter et al. 2006).

27.6.2 Inflammatory Bowel Disease

IBD is believed to develop as a consequence of an altered response to host’s inherent gut mucosal microbiota (German et al. 2003). Changing the luminal microbiota, enhancing the mucosal barrier function, and rectifying the skewed mucosal immune responses with probiotics is thus an attractive treatment modality. Probiotic bacteria are capable of altering the intestinal and systemic immune responses and gut wall epithelial function in humans and in animal models (Fedorak 2008). Currently very little is known whether probiotics would be beneficial in companion animal IBD therapy (German et al. 2003; Trepanier 2009). To the author’s knowledge, no controlled studies evaluating probiotic therapy in dogs or cats with IBD exist. A study investigating the effect of a probiotic mixture in the treatment of food-responsive diarrhea also included a couple of dogs that, based on their clinical findings and mucosal pathology, could have had a diagnosis of IBD as well. However, only mild effects on the gut mucosal immunological parameters were detected after probiotic supplementation in those dogs (Sauter et al. 2006).

27.6.3 Antibiotic-Responsive Diarrhea and Probiotics

Several bacteria (e.g., E. coli, Salmonella enterica spp., Campylobacter jejuni, Clostridium spp., and Yersinia spp.) are known to cause enteritis in humans (DuPont 2009). The role of enteric bacteria as a cause of companion animal chronic or acute diarrhea is controversial. Certain bacteria, such as C. jejuni and C. perfringens, have been suggested to cause enteritis in dogs and cats. Nevertheless, these supposedly pathogenic bacteria have been isolated also in asymptomatic animals, rendering their causative role in diarrhea etiology unclear (Marks and Kather 2003).

Despite the debatable role of enteric bacteria, chronic enteropathies in dogs and cats are often successfully treated with long courses of antibiotics (e.g., a macrolide antibiotic tylosin), thus the term “antibiotic-responsive diarrhea” or “tylosin-responsive diarrhea” has been advocated. However, the prolonged use of antimicrobials may lead to development of antibiotic resistance, and therefore probiotics have been proposed as a substitute to antimicrobials. This concept has been recommended by the World Health Organization (WHO 1997) as an alternative to antibiotics for the prevention and control of production production-related animal diseases.
Dogs found to have tylosin-responsive diarrhea were administered probiotic *L. rhamnosus* GG (LGG) instead of tylosin to treat or prevent the relapse of chronic tylosin-responsive diarrhea. However, LGG supplementation did not prevent the relapse of diarrhea in any of the nine dogs included in the study (Westermarck et al. 2005). LGG is of human origin, which may have influenced the results in the study by Westermarck and co-workers (2005).

### 27.6.4 Giardiasis

Giardiasis is caused by the protozoa *Giardia duodenalis* commonly found in the environment. Reported prevalence in dogs varies from 5% to 35%, and in cats from 2.4% to 60%. *Giardia* is a zoonotic pathogen, and companion animals are suggested to be a reservoir for giardiasis, although this is debated (Gookin et al. 2004; Simpson et al. 2009). Giardiasis is manifested by acute or chronic gastrointestinal signs, mainly diarrhea, and its therapy and prevention may be challenging. Probiotic *E. faecium* SF68 and *L. casei* have been documented to reduce shedding of *Giardia* in mice (Benyacoub et al. 2005), but not in dogs (Simpson et al. 2009).

In gerbils, daily administration of *L. johnsonii* La1 from 7 days before inoculation with *Giardia* trophozoites efficiently prevented *G. intestinalis* infection. Shedding of fecal *Giardia* antigens was diminished in the *L. johnsonii* La1-treated group, and the giardiasis resolved in 21 days. Probiotic administration also protected against parasite-induced mucosal damage, and a cellular response to *Giardia* antigens was stimulated in spleen cells from *L. johnsonii* La1-treated gerbils (Humen et al. 2005).

### 27.7 Atopic Dermatitis

In humans the prevalence of atopic dermatitis (AD) has increased noticeably in the past decades, especially in more industrialized countries. The reason for this is not fully understood, but the “hygiene hypothesis” has been proposed. This hypothesis postulates that a “too clean” childhood environment leads to aberrant Th1/Th2 immune responses, and in the absence of immunological stimulation through certain microbial antigens the body starts to react to environmental antigens normally regarded as harmless. The gut microbiota is believed to play an integral part in the development of AD (Flohr et al. 2005).

AD is one of the most common inflammatory diseases in dogs. It is typically manifested with marked pruritus and dermatitis, which may be chronic or relapsing. Pathogenesis of AD is complex. The affected animals are believed to have a genetic background that causes dysfunction in skin barrier and skewed immunological responses to the environment (Hillier and Griffin 2001). The therapy of AD is based on antigen avoidance, when possible, and modulating the inflammation, for example, by glucocorticoids and cyclosporine. Unfortunately, they may produce remarkable adverse effects especially in chronic use (Olivery et al. 2003). Therefore, attempts to reduce or even replace the need of corticosteroids and cyclosporine are sought after.

Although the exact etiology of AD is unclear, there is evidence to implicate certain type of gut microbiota and altered GALT function with a shift of the Th1/Th2 balance toward a Th2 response. Probiotics have been shown to modulate the immune system back to a Th1 response. Several *in vitro* and *in vivo* studies suggest a role for probiotics in treating allergic disorders. However, many human trials demonstrate a limited benefit for the use of probiotics in prevention or therapy of AD (Kopp and Salfeld 2009).
Probiotic LGG supplementation was shown to be effective in preventing early atopy in children with increased genetic susceptibility (Kalliomäki et al. 2001). In dogs, the genetics is believed to also play a central role in AD pathogenesis, as canine AD is defined as “a genetically predisposed inflammatory and pruritic allergic skin disease with characteristic clinical features associated with IgE antibodies most commonly directed to environmental allergens” (Halliwell 2006).

The effect of LGG supplementation was evaluated in a small-scale study in an experimental setting including two adult female beagles with severe AD and their 16 puppies. Probiotic supplementation was administered to the pregnant bitch and its puppies from 3 weeks to 6 months of age (LGG group); the approximately 1 year older untreated litters from the same bitches served as controls. All puppies were sensitized to a common canine allergen, *Dermatophagoides farinae*, and blood levels of allergen-specific IgE together with clinical signs of AD were followed up. Administration of LGG reduced the allergen-specific IgE concentrations significantly when compared with untreated litter. Additionally, intradermal allergen test scores showed significantly milder reactivity in LGG group. No significant effect on the clinical signs was noted. The study suggests that probiotics may have a beneficial effect in alleviating the development of AD in genetically predisposed puppies. Further studies with a larger number of dogs and in a clinical setting is needed to further evaluate this potentially promising new therapy modality in AD dogs (Marsella 2008).

### 27.8 Other Probiotic-Induced Immunological Effects in Companion Animals

*E. faecium* SF68 has been documented to enhance specific immunological responses in young dogs. It increased the fecal and serum immunoglobulin A concentration, increased immune response to canine distemper virus vaccination, and increased the percentage of circulating B lymphocytes when compared with the control group (Benyacoub et al. 2003). A similar study was performed on kittens with the hypothesis that *E. faecium* SF68 supplementation would enhance vaccine-related immune responses. However, feeding *E. faecium* SF68 did not alter most of the immune parameters evaluated in the study. The increased CD4+ cell numbers could have been nonspecific (Veir et al. 2007).

A single oral supplementation of *E. faecalis* FK-23 at a dose of 100 mg/kg stimulated nonspecific immune responses in healthy adult dogs. In the study, a complete blood count (CBC), leukocyte differential count, phagocytic activity of peripheral blood neutrophils, and a lymphocyte blast transformation (LBT) test were assessed.

*E. faecalis* FK-23 had no effect on the CBC or leukocyte differential count. However, FK-23 supplementation resulted in 1.4-fold increase in neutrophil phagocytosis compared with the control group. The FK-23 administration led to a twofold increase of LBT activities of phytohemagglutinin and pokeweed mitogen when compared with the control group. Activation of concanavalin A was increased to 150%. It was concluded that a single administration of *E. faecalis* FK-23 enhanced host resistance through stimulation of the nonspecific immune responses *in vivo* (Kanasugi et al. 1997).

### 27.9 Probiotic Effects of Canine Vaginal Microbiota

LAB are common habitants of canine vaginal mucosa. Disturbances in vaginal microbiological homeostasis may lead to bacterial vaginitis, or other genital and reproductive problems.
A study involving 42 bitches evaluated the relationship of vaginal mucosal LAB in certain clinical conditions, and furthermore assessed the potential probiotic properties of selected bacteria. From the cultured vaginal swabs, *L. murinus*, *L. plantarum*, and *E. canintestini* were chosen for further studies. All selected strains showed antimicrobial activity and ability to adhere to canine vaginal epithelial cells *in vitro*, although the adherence varied significantly, *Lactobacillus* sp. 12C1 being the most adhesive strain. The possible clinical exploitation of the isolated strains needs to be further studied (Delucchi et al. 2008).

### 27.10 Prevention of Calcium Oxalate Urolithiasis with Probiotics

Calcium oxalate urinary tract stones are a common clinical finding in both cats and dogs. Removal, usually by surgery or urohydropulsion for smaller stones, is the only successful treatment (Bartges et al. 2004). Hypercalciuria and particularly hyperoxaluria are considered to be the major factors in calcium oxalate urolithiasis formation (Weese et al. 2004; Murphy et al. 2009). Oxalate excretion to urine is related to dietary intake. Dietary modification is the cornerstone for preventing urolithiasis formation; however, calcium oxalate urinary stones are often recurring (Bartges et al. 2004).

Intestinal microbiota is known to have a marked effect on intestinal levels of oxalate. The oxalate-degrading bacterium *Oxalobacter formigenes* has been isolated from rat and human intestines, and its presence is related to lower risk of hyperoxaluria (Troxel et al. 2003). In addition, LAB isolated from canine feces were capable of degrading intestinal oxalates and reduce oxaluria (Weese et al. 2004; Murphy et al. 2009). The LAB were identified as *Leuconostoc lactis* (two strains), *L. acidophilus* (two strains), and one strain of *Lactobacillus plantarum* (Weese et al. 2004).

In another study, *Lactobacillus animalis* 5323 and *L. animalis* 223C reduced urine oxalate concentrations significantly. Modifying intestinal microbiota may provide new tools for calcium oxalate therapy in companion animals, but the candidate strains need to be tested in vivo as *in vitro* testing may not indicate an *in vivo* activity (Murphy et al. 2009).

### 27.11 Conclusion

There is a keen interest of utilizing probiotics in companion animal nutrition and medicine among veterinarians, animal nutritionists, and pet owners. While there is some evidence that certain probiotic strains may be useful in feline and canine welfare and medical therapy, there is also an obvious call for more controlled clinical studies to enlighten the potential role of probiotics in companion animals. Whether the existing commercial human probiotics are able to exert their documented beneficial health effects also in dogs and cats, or whether the most suitable probiotics should be species specific, should be evaluated as well.

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Chapter 28

Prevalence and Application of Lactic Acid Bacteria in Aquatic Environments

Hélène L. Lauzon and Einar Ringø

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28.1 Introduction

Lactic acid bacteria (LAB) are widespread in nature, originating from soil, water, plants and animals. LAB include several genera that do not form a monophyletic group, but should rather be considered as a biologically functional group with diverse properties. Various compounds are formed during lactic fermentation of LAB, such as organic acids, diacetyl, hydrogen peroxide, and bacteriocin or bactericidal proteins (Vandenbergh 1993). Indeed, LAB have been used in fermentation to preserve food and enhance their characteristics, as protective cultures to control foodborne pathogens or as probiotics to enhance human and livestock health (Stiles 1996; Giraffa et al. 2010; Leroi 2010). LAB application in the field of aquaculture is a growing area of probiotic research (Ringø and Gatesoupe 1998; Ringø 2004; Ringø et al. 2005; Balcazar et al. 2006; Gatesoupe 2008; Merrifield et al. 2010b). The excellent safety record of several LAB species makes them an attractive solution as biological agents. However, LAB pathogenic to aquatic animals have been reported among *Carnobacterium*, *Lactococcus*, *Streptococcus*, *Vagococcus*, and *Weissella* genera. *Streptococcus iniae* and *Lactococcus garvieae* are true pathogenic species of worldwide significance against which commercial vaccines are available (Gatesoupe 2008). Warm water infections (>15°C) caused by *Lac. garvieae*, *Strep. iniae*, *Strep. difficile*, and *Strep. parauberis* occur in both cultured freshwater and marine fish, while coldwater infections (<15°C) of salmonids are caused by *Vagococcus salmoninarum* and *Lac. piscium* (Muzquiz et al. 1999). *Carnobacterium maltaromaticum* and related species were suggested to be fish pathogens (Leisner et al. 2007; Loch et al. 2008). Recently, Liu et al. (2009) reported a novel species of *Weissella* as an opportunistic pathogen for rainbow trout (*Oncorhynchus mykiss* Walbaum) in China. Nevertheless, LAB are generally harmless, with some strains demonstrating beneficial effects on fish health (Gatesoupe 2008).

28.2 Prevalence of LAB in Aquatic Animals and Environments

LAB are anaero-aerotolerant and found in several niches, indicating their adaptive characteristic, but they generally have complex nutritional requirements (Leroi 2010). Recent interest in the presence of LAB in aquatic animals has been unveiled by the numerous studies published in the last 5 years. However, less emphasis has been put toward an overall ecological understanding since little information is available on the prevalence of LAB in aquatic environments. This search for autochthonous LAB in aquatic animals most often aims to define putative probionts from different rearing environments and to design tailor-made solutions. In that respect, more attention has been paid to the isolation media and conditions used to successfully retrieve LAB specific to each environment. Further, advances in molecular techniques and their wider availability have contributed to a better identification of LAB isolates. A study, genotypically analyzing 57 LAB strains of different fish origins, recognized the considerable diversity of LAB associated with fish, which may explain the difficulties encountered in earlier years during phenotypical identification. They also confirmed the repeated associations of *Lb. raffinolactis*, *Aerococcus* spp., *Enterococcus* spp., and *Vagococcus* spp. with apparently healthy fish. Finally they enunciated that a variety of LAB, as yet unsuspected or poorly documented, colonize live fish either regularly or transiently (Michel et al. 2007).

28.2.1 LAB in Aquatic Animals

The close relation of aquatic animals to their external environment suggests that uptake of microorganisms via osmoregulation and feeding processes will influence the microbiota of the skin,
gills, and gastrointestinal (GI) tract. This is true at early stages of life (Hansen and Olafsen 1989), but it has been observed that the environmental microbiota is not necessarily the one to become established in aquatic animals at a later stage. Further, environmental fluctuations may lead to variations in microbiota. This can easily be foreseen since aquatic animals are poikilothermic and a change in temperature may modify their microbiota (Lésel 1990). Water salinity and diets are also primary factors influencing the gut microbiota of fish (Ringø and Strøm 1994). Table 28.1 presents numerous studies where LAB were confirmed as part of the microbiota of aquatic animals, with most publications reporting on culture based methods using various microbiological media incubated over a wide temperature range (12–37°C). Emphasis has been toward the analysis of the GI tract, while little information relating to LAB of skin and gills is available (Ringø and Holzapfel 2000).

### 28.2.1.1 Prevalence of LAB on Skin and Gills

A first study to report on the presence of LAB on gills of reared adult Atlantic salmon (*Salmo salar* L.) isolated carnobacteria (26%) among the microbiota studied, suggesting them as members of the normal microbiota from this marine environment (Ringø and Holzapfel 2000). Further strain characterization revealed the potential antagonistic properties of these *C. piscicola* maltaromaticum–like isolates toward three fish pathogens (*Aeromonas salmonicida* ssp. *salmonicida*, *Vibrio [Listonella] anguillarum*, and *Aliivibrio [Vibrio] salmonicida*). Gonzalez et al. (1999) examined the microbiota of wild brown trout (*Salmo trutta*), pike (*Esox lucius*), and farmed rainbow trout, analyzing their skin, gills, intestinal tract, and environmental water. They retrieved several *Lactobacillus*, *Carnobacterium*, *Enterococcus*, and *Lactococcus* spp. as well as *Vag. fluvialis* from the different samples analyzed (Gonzalez et al. 2000). Microbiota of wild brown trout gills and skin was dominated (44–50%) by *Carnobacterium* spp., which were also isolated (20%) from wild pike’s gills as well as *Lactococcus* spp. (10%). Farmed rainbow trout harbored *Enterococcus* spp. (10%) on their skin and *Lactobacillus* spp. (11%) on their gills despite the presence of *Carnobacterium* spp. (14%) in the water (Gonzalez et al. 1999). *Streptococcus* spp. were isolated from the intestine, sediment and rearing water of hybrid tilapia (*Oreochromis niloticus × O. aureus*) at low levels (around 1% of culturable microbiota) but not on their gills (Al-Harbi and Uddin 2003). The microbiota on the ventral skin of bullfrog (*Rana catesbeiana*) larvae at different seasons has been studied in Argentinian hatcheries. *Lactobacillus* and *Pediococcus* spp. prevailed in healthy individuals in the autumn (Pasteris et al. 2006), while low levels of *Enterococcus faecalis*, *Ent. faecium*, and *Streptococcus* spp. were also isolated during spring and summer periods (Pasteris et al. 2009). This limited information does not allow for a comprehensive evaluation of LAB prevalence on skin and gills of aquatic animals from different environments, but a greater diversity may occur in freshwater species.

It is noteworthy that *in vitro* studies have demonstrated the ability of LAB to adhere to intestinal mucus of different origins, namely from human, canine, possum, bird, and fish sources (Rinkinen et al. 2003b). Similarly, Nikoskelainen et al. (2001b) showed that human- and dairy-derived LAB strains could adhere to different fish mucus types, originating from the esophagus, stomach, intestine, skin, and gills. This suggests that such LAB adhesion is nonspecific, that is, bacteria may bind to the mucosal surface by van der Waals and electrostatic forces, hydrophobic interactions, or hydrogen bonds. In contrast, specific adhesion is mediated by bacterial adhesins and corresponding receptors at the mucosal site (Rinkinen 2004). Adhesion of LAB strains to mucosae may imply both mechanisms (von Ossowski et al. 2010). Environmental factors certainly influence bacterial adhesion. Higher shear rates, like flow of water, will promote bacterial
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(continued)
Table 28.1 LAB Isolated from Aquatic Animals (Continued)

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<td>Atlantic salmon (F)</td>
<td>A</td>
<td>PI/DI/ ns sakei</td>
<td>Askarian et al. (2011b)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Atlantic salmon (F)</td>
<td>A</td>
<td>I/D DGGE</td>
<td>Hovda et al. (2007)</td>
<td></td>
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<tr>
<td>Atlantic salmon</td>
<td>A</td>
<td>I</td>
<td>Strom (1968)</td>
<td></td>
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<tr>
<td>Atlantic salmon</td>
<td>A</td>
<td>I</td>
<td>(1), (3), (4)</td>
<td></td>
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<td></td>
<td></td>
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</tbody>
</table>

Note: F, farmed; W, wild; L, larvae; J, juvenile; A, adult; IM, immunostimulated; GIT, gastrointestinal tract (including digesta); D, digesta only (allochthonous bacteria); St, stomach; PC, pyloric ceca; PI, proximal intestine; DI, distal intestine; HC, hindgut chamber wall; HCC, hindgut chamber content; I, intestinal wall (autochthonous bacteria); M, muscle; S, skin; G, gills; RW, rearing water; all, allochthonous bacteria; aut, autochthonous bacteria; s, summer; w, winter; sp/f, spring and fall; ns, not specified (species not defined); CLONE, culture-independent analysis using 16S rDNA clone libraries; DGGE, culture-independent analysis using PCR and denaturing gradient gel electrophoresis; (1) Strom and Ringø, unpublished; (2) Ringø, unpublished; (3) Ringø and Sperstad, unpublished; (4) Ringø and Bendiksen, unpublished.
detachment, while ionic strength and pH influence bacterial adhesion by changing surface characteristics of both the bacteria and the materials, with higher ionic strength and lower pH contributing to better adhesion (Katsikogianni and Missirlis 2004). It is therefore concluded that LAB may adhere to skin and gills of aquatic animals, but environmental conditions will be determinative.

28.2.1.2 Prevalence of LAB in Digestive Tract

The composition of the microbiota in the digestive tract (DT) of fish has been reported to vary according to developmental stages, diet, environmental conditions, and stress factors, as reviewed by Nayak (2010b). Levels of bacterial loads have been observed to vary with developmental stages as well as the different DT regions. Several LAB species have been isolated from the DT of aquatic animals. Although they do not generally prevail among the gut microbiota, their abundance in freshwater fish has been notably higher than in marine species (Gatesoupe 2008). Table 28.1 lists several studies evaluating the prevalence of LAB in the DT of aquatic animals at different developmental stages from a natural (wild) or farmed environment. Most of the studies evaluated the allochthonous (transient) microbiota, while some of them examined the autochthonous (adherent) bacteria colonizing the mucus layer and/or the epithelial surface of the DT.

Overall it is observed from Table 28.1 that marine species from temperate and coldwater areas have a higher prevalence of *Carnobacterium* spp. along with *Lactobacillus*, *Enterococcus*, and *Streptococcus* spp., while those from warm water zones harbor *Lactobacillus*, *Leuconostoc*, and *Weissella* spp. mainly, as well as *Lactococcus* and *Enterococcus* spp., but to a lesser extent. *Lactobacillus*, *Enterococcus*, *Lactococcus*, *Leuconostoc* and *Streptococcus* spp. are frequently isolated from freshwater species, with *Carnobacterium* spp. being of greater importance in those from temperate waters. These observations agree with the analysis conducted by Izvekova et al. (2007) on published data dealing with the taxonomic composition of the intestinal microbiota in fish living under different conditions and feeding differently, which led to the conclusion that the composition of intestinal microbiota is influenced by fish ecology. They reported the presence of *Lactobacillus* spp. in both marine and freshwater species, whereas *Streptococcus*, *Carnobacterium*, *Lactococcus*, *Vagococcus* spp., and *Ent. durans* were only attributed to freshwater species. LAB prevalence was mainly associated with predators, but *Lactobacillus* spp. were also present in planktophages, benthophages, and euryphages while *Streptococcus* spp. were isolated from herbivores and euryphages as well. Bucio et al. (2006) isolated *Lactobacillus* spp. in freshwater fish with several feeding habits: carnivorous, such as perch (*Perca fluviatilis*), European eel (*Anguilla anguilla*), and catfish (*Silurus glanis*); herbivorous, such as carp (*Abramis brama*); and omnivorous, such as rudd (*Scardinius erythrophthalmus*). Indeed as pointed out by our data assessment, the wide occurrence of *Lactobacillus* spp. throughout these different aquatic environments may reflect the ubiquitous nature of this genus. It is in fact the largest genus of the *Lactobacillaceae* family, with over 100 species and subspecies (Giraffa et al. 2010).

The gut microbiota has been shown to be influenced by seasonal variations. MacMillan and Santucci (1990) observed such variations in the intestine of farm-raised channel catfish (*Ictalurus punctatus*) with respect to nonenterococcal group D streptococci among the dominating gram-positive microbiota. Bucio et al. (2006) reported variations in lactobacilli load of digesta from river fish (perch, carp, and rudd), being highest in summer and lowest in winter. Several LAB species contributed to bullfrog hatchery microbiota and its population varied according to the season, the area of the hatchery, as well as the health status of the animals (Pasteris et al. 2006; Pasteris et al. 2009). Investigating the digesta microbiota of silver carp (*Hypophthalmichthys molitrix*), common carp (*Cyprinus carpio*), channel catfish, and deepbodied crucian carp (*Carassius cuvieri*), Hagi et
al. (2004) observed a shift from \textit{Lac. raffinolactis} in winter to \textit{Lac. lactis} in summer. \textit{C. maltaromaticum} and \textit{Streptococcus} spp. were associated with some winter samples while \textit{Lb. curvatus} and \textit{Ent. pseudoavium} were present at intermediate phases. Recently, Sica et al. (2010) analyzed the allochthonous intestinal microbiota of wild Jenyn’s sprat (\textit{Ramnogaster arcuata}) caught in summer (22°C) and winter (9°C) on the coast of Bahía Blanca Estuary (southwest Atlantic Ocean). \textit{Weissella viridescens} was present during both seasons, while \textit{Leuc. citreum} was isolated in winter but \textit{Lb. paracasei} ssp. \textit{tolerans} and \textit{Ent. hirae} in summer. Other fish species examined in wintertime harbored \textit{Leuc. mesenteroides} ssp. \textit{mesenteroides} as well.

\subsection*{28.2.1.3 Factors Affecting LAB in the DT}

It is generally accepted that dietary changes modulate the autochthonous (mucosal) gut microbiota of fish (Ringø et al. in prep.). Readers with special interest regarding the effect of chromic oxide (Cr$_2$O$_3$), dietary lipid levels, lipid sources, polyunsaturated fatty acids, prebiotics, stress, and salinity on LAB in the GI tract are referred to the comprehensive review of Ringø and Gatesoupe (1998), Ringø (2004), Merrifield et al. (2010b), and Ringø et al. (2010b).

\subsection*{28.2.2 Other Aquatic Habitats of LAB}

LAB have not been commonly associated with the aquatic environment, which could be explained by the cultivation methods applied. Their prevalence in endothermic animals and the cultivation methodology associated with their isolation from such sources may have contributed to their low retrieval in other environments with different requirements. Being nutritionally fastidious and adapting to various environments, LAB may require special cultivation media with appropriate incubation temperature and time (Ringø and Gatesoupe 1998). The use of a single LAB medium may not allow for the isolation of all culturable LAB species. However, recent studies have indicated the prevalence of several LAB genera in habitats from aquatic environments, natural and aquacultural.

\subsection*{28.2.2.1 Natural Environment}

Studies dealing with microbiota of diverse aquatic habitats have not reported a clear prevalence of LAB (Sfanos et al. 2005), perhaps due to a cultivation-related biased outcome. However, some culture-independent studies also indicate a very low LAB prevalence or absence (Kormas et al. 2003; Kim et al. 2004; Lee et al. 2004; Wang et al. 2010; Lachnit et al. 2011). Nonetheless, isolation of two new genera of slightly halophilic and alkaliophilic LAB, \textit{Marinilactibacillus} and \textit{Halolactibacillus}, was achieved during the examination of living and decaying marine organisms from temperate and subtropical areas of Japan, together with three novel species: \textit{Mar. psychrotolerans} (Ishikawa et al. 2003), \textit{Hal. halophilus}, and \textit{Hal. miurensis} (Ishikawa et al. 2005). Further, analysis of marine deep sediment samples from different areas has revealed the presence of \textit{Carnobacterium} and \textit{Marinilactibacillus} spp. (Newberry et al. 2004; Toffin et al. 2004; Parkes et al. 2009) and its novel species \textit{Mar. piezotolerans} (Toffin et al. 2005). A \textit{Marinilactibacillus} sp. was one of the isolates obtained from the sediments underlying the oxygen minimum zone of the Arabian Sea (Divya et al. 2010). Several common LAB species (\textit{Lb. pentosus}, \textit{Lb. graminis}, \textit{Ent. mundii}, \textit{Ent. durans}, \textit{Pediococcus pentosaceus}, \textit{W. viridescens}) were detected in the surface sediment of an Argentinian estuary (Sica et al. 2010). Evaluation of sediment from the north coast of Mexico confirmed the presence of \textit{Weissella} spp. (likely \textit{W. cibaria}), \textit{Ent. faecium}, and \textit{Lac. garvieae}.
Lactic Acid Bacteria: Microbiological and Functional Aspects

(Zamudio-Maya et al. 2008). Novel psychrotrophic *Carnobacterium* spp., *C. funditum*, and *C. alterfunditum*, were isolated from anoxic Antarctic lake water (Franzmann et al. 1991). Recently, Vela et al. (2008) reported a novel *Lactobacillus* species, *Lb. ceti*, isolated from the lungs and liver of beaked whales (*Ziphius cavirostris*).

In freshwater environments, the prevalence of several LAB genera has generally been reported. *Lactobacillus aquaticus* was first retrieved from a Korean freshwater pond (Song et al. 2007; Manes-Lazaro et al. 2009). In Kenya, isolation of *Enterococcus*, *Alkalibacterium*, and *Marinilactibacillus* spp. from a haloalkaline lake was achieved (Mwirichia et al. 2010). Gonzalez et al. (1999) reported the prevalence of *Carnobacterium* and *Lactobacillus* spp., 20% and 10%, respectively, in water samples obtained from nine rivers in the northwest region of Spain. LAB obtained from water of seven lakes in Japan were represented by seven genera: *Lactococcus*, *Leuconostoc*, *Enterococcus*, *Lactobacillus*, *Carnobacterium*, *Streptococcus*, and *Weissella*, with *Lac. lactis* ssp. *lactis* being the most abundant species isolated throughout the year. Seasonal differences were recorded (Yanagida et al. 2007). Overall six LAB genera were common to both freshwater and marine environments: *Lactobacillus*, *Enterococcus*, *Carnobacterium*, *Lactococcus*, *Weissella*, and *Marinilactibacillus*.

### 28.2.2.2 Aquaculture Environment

Occurrence of LAB in aquacultural units and their related components has been documented in few studies. Rearing water analysis by cultivation has revealed *Carnobacterium* spp. (14% of total culturable microbiota) in rainbow trout rearing (Gonzalez et al. 1999); *Ent. faecalis*, *Lactobacillus*, and *Pediococcus* spp. in bullfrog rearing in addition to *Ent. faecium* in their feed (Pasteris et al. 2009); and *Lactobacillus* spp. in shrimp rearing (Ma et al. 2009). Lauzon et al. (2010a) reported the presence of *Ent. thailandicus*, *Ent. pseudoavium*, and *Lac. lactis* ssp. *lactis* in dry feed used for Atlantic cod (*Gadus morhua* L.) rearing. They also isolated strains of *Ent. phoeniculicola*, *Ent. pseudoavium*, *Alkalibacterium putridalgicola*, and *Alk. olivoapovliticus* from an algal concentrate added to the rearing water. Two LAB strains, tentatively identified as *Lb. plantarum* and *Carnobacterium* sp., were isolated from a rotifer (*Brachionus plicatilis*) culture (Gatesoupe 1994). Further, a strain of *Lac. lactis* with inhibitory effect against a *V. anguillarum* strain was obtained from a rotifer mass culture (Harzevili et al. 1998).

### 28.3 Application of LAB as Probiotics in Aquaculture

Research on isolation and application of probiotics in aquaculture started more than two decades ago. The main reason for the search of probiotics was the need for alternative prophylactic methods to deal with the survival problems encountered at different stages of intensive rearing. Proliferation of opportunistic and pathogenic microorganisms is known to cause poor larval growth and high mortality rates (Munro et al. 1995). As a result, juvenile availability and quality is the main bottleneck for several of the different aquatic species reared. Antibiotics have been widely used for prevention and control of aquaculture diseases, especially at early stages where vaccination is not possible, but a growing fear for the emergence of resistant bacteria and environmental problems has called for novel and environmentally friendly solutions (Verschuere et al. 2000; Serrano 2005).

Fuller (1989) refers to a probiotic as a live microbial feed supplement that beneficially affects the host animal by improving its intestinal microbial balance. Indeed, the DT is a major route of entry for pathogens. However, the situation for aquatic animals differs from that for humans and livestock since, in addition to the intestinal tract, other routes of entry for pathogens, such as gills...
and skin, play a role in infection (Ringø et al. 2007a; Engelsen et al. 2008). In that respect, the
definition of a probiotic enunciated by Verschuere et al. (2000) is relevant in aquaculture applica-
tion: a live microbial adjunct that benefits the host by modifying the host-associated or ambient
microbial community, by enhancing the host response toward disease, by ensuring improved use
of feed or enhancing its nutritional value or by improving the quality of its ambient environment.
This suggests that probiotic transmission to the host can be achieved by two vectors, either via
the diet or the rearing water, as proposed by Gatesoupe (1999). It has also been proposed that the
application of a live probiotic is not a prerequisite since use of inactivated cells has led to positive
health effects (Ouwehand and Salminen 1998). In fact, Salminen et al. (1999) defined probiotics
as “microbial cell preparations or components of microbial cells that have a beneficial effect on
the health and well-being of the host.” This implies that bacterial metabolites and bacteria-derived
immunostimulants may then be considered as probiotics.

Many probiotics tested in aquatic animals have been selected due to their efficiency in humans
and livestock (Azad and Ai-Marzouk 2008). Table 28.2 lists commercially available LAB-
containing products claiming to have probiotic properties in aquatic environments. The specific
uses (rearing water or feed for some aquatic animals) and stages of introduction (larvae or juveniles)
apparently restrict the possible application of these LAB probiotics. It is noteworthy that
five of them (Bactocell®, Alchem Poseidon, Efino® L, Sorbiflore, and Cernivet® LBC ME10) have
been reported in peer-reviewed studies. Because of its proven safety and efficacy in beneficially
enhancing production, Bactocell® is the first zootechnical aquaculture feed additive authorized
in Europe for salmonids and shrimp (http://lallemandanimalnutrition.com/category/markets/
europe). It has been used since 2005 in feed for fattening pigs and chickens.

It is therefore observed that very few commercial solutions are available, targeting a restricted
number of aquatic species. This may be explained by the fact that the main efforts being placed
in aquaculture research dealing with the identification and testing of probiotics in contrast to the
lack of upscaling feasibility, long-term safety, and efficiency testing as well as industrial produc-
tion of new probiotic solutions. Further, current legislation may hamper a desirable development
in that urgently needed area of work.

28.3.1 Probiont Selection: What Should Be Considered?
The selection of potential probiotics is a complex task since several factors may affect their efficacy
upon their application in rearing environments. Environmental conditions (pH, temperature,
salinity, nutrients, microbial competition) will inevitably influence the success of probiotics. It is
therefore important to obtain a better understanding of LAB requirements for growth and mainte-
nance in aquatic environments. The criteria for probiont selection and application should consider
their origin, the targeted properties that must be fulfilled, the form and method of application
required, as well as their effects and safety to the host, and alternatively humans. These character-
istics have been proposed to define promising candidates, with some of them being essential (safe
and environmentally tolerant) and others considered favorable criteria (Merrifield et al. 2010b).

28.3.1.1 Origin of Strains and Host Specificity
Testing of probiotics in aquaculture has to some extent been based on their effects in humans
(Nikoskelainen et al. 2001a; Panigrahi et al. 2004; Balcazar et al. 2007a) and livestock (Gatesoupe
2002; El-Haroun et al. 2006; Suzer et al. 2008). This approach is supported by the proven safety
for human consumption, as the aquatic animals will ultimately become foods. However, their
Table 28.2 Commercial LAB-Containing “Probiotic” Products for Aquaculture, Their Application Purpose, Benefits, and Microbial Content

<table>
<thead>
<tr>
<th>Product (Company)</th>
<th>Application</th>
<th>Benefits Mentioned</th>
<th>Microbial Content</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>TOARAZE (TOA Pharmaceutical Company of Japan)</td>
<td>mixed to feed for eel and shrimp</td>
<td>inhibits GIT pathogens; improves feed effectiveness, growth rate and water quality; increases farming yield</td>
<td>Enterococcus faecalis T-110, Clostridium butyricum TO-A, Bacillus mesentericus TO-A</td>
<td>a</td>
</tr>
<tr>
<td>Progut (Bio Ops)</td>
<td>gut probiotic</td>
<td></td>
<td>several LAB, Bacillus and Aspergillus spp., Trichoderma reesei</td>
<td>b</td>
</tr>
<tr>
<td>Pro-3000 series (Aqua-in-tech)</td>
<td>water or feed</td>
<td>reduces stress and improves survival rates</td>
<td>B. subtilis, B. licheniformis, Lactobacillus acidophilus, and Saccharomyces cerevisiae</td>
<td>c</td>
</tr>
<tr>
<td>BACTOCELL® (Lallemand)</td>
<td>feed additive for salmonids, shrimp</td>
<td>enhances salmonids and shrimp production</td>
<td>Pediococcus acidilactici CNCM MA18/5M</td>
<td>d</td>
</tr>
<tr>
<td>Alchem Poseidon (Alchem Korea)</td>
<td>water or feed addition</td>
<td>enhances nonspecific immune parameters</td>
<td>B. subtilis (&gt;1.6 × 10^7 cfu/g), Lb. acidophilus (&gt;1.2 × 10^8 cfu/g), Cl. butyricum (&gt;2.0 × 10^7 cfu/g) and S. cerevisiae (&gt;1.6 × 10^7 cfu/g)</td>
<td>e</td>
</tr>
<tr>
<td>Efinol® PT Bentoli Inc.</td>
<td>microbial culture for shrimp and fish ponds; feed coating</td>
<td>lowers stress effects; minimizes mortality during disease outbreak; improves uniformity and yield of aquatic species; helps maintain favorable microbial balance and water quality conditions in ponds</td>
<td>B. subtilis, B. licheniformis, B. coagulans, Lb. acidophilus, Streptococcus faecium, S. cerevisiae</td>
<td>f</td>
</tr>
</tbody>
</table>

(continued)
Prevalence and Application of Lactic Acid Bacteria in Aquatic Environments

efficacy in aquatic environments may vary due to the different conditions inherent to each environment. For instance, mesophilic LAB may not perform as well in a colder aquatic environment, which may be saline or even alkaline. Indeed, Vazquez et al. (2003) reported a short survival time (half-life = 3–21 h) for allochthonous LAB in seawater at 20–30°C.

Further, host specificity may be an important factor to consider for long-lasting probiont colonization. According to Conway (1996), a microorganism is able to colonize the GI tract when it can persist there for a long time, by possessing a multiplication rate higher than the expulsion rate. Later, Nikoskelainen et al. (2001b) suggested that mucosal adhesion is one of the five important criteria for selection of probiotics in fish. However, in their recent review devoted to probiotic and prebiotic applications for salmonids, Merrifield et al. (2010b) proposed an extended list of 11 essential and favorable criteria for potential probiotics, and the authors proposed that probiotic colonization of the intestinal epithelial surface is a favorable criterion. Whereas some authors suggested that LAB colonization of intestinal mucus involves host specificity (Lin and Savage 1984; Fuller 1986; Askarian et al. 2011a; Salma et al. 2011), other authors reported the absence of

Table 28.2 Commercial LAB-Containing “Probiotic” Products for Aquaculture, Their Application Purpose, Benefits, and Microbial Content (Continued)

<table>
<thead>
<tr>
<th>Product (Company)</th>
<th>Application</th>
<th>Benefits Mentioned</th>
<th>Microbial Content</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Efinol® L (Bentoli Inc.)</td>
<td>water addition</td>
<td>reduces stress; improves survival rates; produce stronger and healthier larvae and post-larvae</td>
<td>B. subtilis, B. licheniformis, Lb. acidophilus, S. cerevisiae</td>
<td></td>
</tr>
<tr>
<td>SORBIFLORE (Danisco France SAS)</td>
<td>feed addition; for piglets</td>
<td>reduces malformation in sea bass juveniles, increases survival, activates digestive enzymes</td>
<td>Lb. farcininis MA27/6R (CNCM-I-3699), Lb. rhamnosus MA27/6B (CNCM-I-3698)</td>
<td>g</td>
</tr>
<tr>
<td>Cernivet® LBC ME10 (Cerbios-Pharma SA)</td>
<td>pelletable microbial feed additive for livestock</td>
<td>stabilizes intestinal microbiota based on probiotic active ingredient; increases disease resistance toward Edwardsiellosis in eels</td>
<td>Enterococcus faecium SF68®</td>
<td>h</td>
</tr>
</tbody>
</table>

Note: All website information accessed February 11, 2010; a: http://bio-three.com/aqua.php; b: http://www.somphyto.com/probiotics_proaqua.htm; c: http://aqua-in-tech.com/probiotics%20cover.htm; d: http://lallemandanimalnutrition.com/category/markets/europe; (Gatesoupe 2002); e: Probiotics via feed (live or dead cells) or supplying live cells to the tilapia rearing water in a closed recirculating system (Taoka et al. 2006); f: http://bentoli.biz.efeedlink.com/Products/s6110-0c3d3749-2d6b-4b91-a384-767da3704658.html (Comes et al. 2008; Gomes et al. 2009); g: Probiotics via feed (live or dead cells) (Frouel et al. 2008); h: Probiotics via feed (Chang and Liu 2002).
specificity when binding host intestinal epithelial surface (Gildberg and Mikkelsen 1998; Ringø 1999; Rinkinen et al. 2003b; Salinas et al. 2008b). Nevertheless, host specificity can be advantageous since bacteria relying on specific adherence mechanisms may more easily compete with or displace pathogens and prevent their own washout. This is why research has focused on the isolation and selection of autochthonous bacteria, that is, those originating from the respective aquatic animals (Gatesoupe 1999; Azad and Al-Marzouk 2008).

28.3.1.2 Targeted Properties

Several characteristics have been considered to define putative probionts. Inhibition of pathogen growth and adhesion, tolerance to environmental conditions (bile, pH, salinity, temperature), growth characteristics, metabolite production, mucosal adhesion, and immune modulation are widely used criteria (Verschuere et al. 2000; Balcazar et al. 2006; Vine et al. 2006; Gomez and Balcazar 2008). Less frequently evaluated characteristics include inhibition of virulence gene expression or disruption of quorum sensing, improvement of water quality, and enzymatic contribution to digestion. Recently, molecular techniques have brought the evaluation to the gene expression level, targeting markers for immune response, stress response, growth factors, welfare, and metabolism (Carnevali et al. 2006; Rollo et al. 2006; Reyes-Becerril et al. 2008; Avella et al. 2010).

The probiont’s ability to withstand environmental conditions and eventually grow are key characteristics to ensure its survival and maintenance, which are the prerequisites for mucosal adhesion. This is especially true when probionts have the ability to grow faster than other opportunistic or pathogenic bacteria. Lag time and doubling time are important criteria for the comparison of bacterial growth curves (Vine et al. 2004). Adhesion capacity is an important criterion for probiont selection since adherence to the host GI tract is the first step in a probiotic treatment potentially leading to transient colonization, stimulation of the immune system, and antagonistic activity against enteropathogens in situ. In turn, pathogen antagonism is achieved by production of inhibitory compounds, and competition for nutrients or site of attachment. One of the most important beneficial health claims of probiotic bacteria is competitive exclusion of pathogens (Rinkinen et al. 2003a).

During the last decade, numerous studies have been conducted demonstrating that probiotics, including LAB, have an effect on gut enzyme activities of various fish species. Readers with special interest regarding this topic are referred to the comprehensive review of Ray et al. (2011).

28.3.1.3 LAB versus Pathogens in the GI Tract

It is well known that the GI tract of fish is an important portal for bacterial infection (Groff and Lapatra 2000; Birkbeck and Ringø 2005; Harikrishnan and Balasundaram 2005; Ringø et al. 2007a; Ringø et al. 2007b; Salinas et al. 2008b), and it has been reported that an essential and prerequisite step for bacterial invasion is the translocation of bacteria across the intestine (Ringø et al. 2007a). However, this is difficult to study effectively in vivo and such studies are time consuming and expensive. According to the European Union (EU) directive (Directive 86/609/EEC; September 8, 2010), it is recommended to reduce the numbers of in vivo experiments and experimental fishes. During the last decade, three different ex vivo methods (the Ussing chamber, everted sack, and intestinal sack) have been carried out to fulfill the instructions given by the EU. These methods have been used to evaluate translocation and cell damage caused by pathogenic bacteria (Ringø et al. 2004; Løvmo 2007; Ringø et al. 2007a; Ringø et al. 2007b; Jutfelt et al.
2008; Salinas et al. 2008b; Khemiss et al. 2009; Ringø et al. 2010a; Løvmo Martinsen et al. 2011; Salma et al. 2011). The major aim of the studies using the intestinal sack method was to assess whether LAB is able to adhere to and colonize the fish intestine and outcompete V. anguillarum and Aer. salmonicida (Løvmo 2007; Ringø et al. 2007b; Salinas et al. 2008b; Ringø et al. 2010a; Løvmo Martinsen et al. 2011; Salma et al. 2011). The conclusion from these studies is that the LAB are to some extent able to outcompete the pathogen. However, further studies are needed using polymerase chain reaction–denaturing gradient gel electrophoresis (PCR-DGGE), specific probes for FISH, specific primers for LAB DGGE, and immunogold labeling.

28.3.1.4 Probiotic Form of Application

The viability of probiotics at application is a current topic of discussion, as further research in that area will provide an insight into their mode of action. For practicality and ease of standardization, the possible use of freeze-dried and inactivated cells may contribute to the commercialization of probiotics. However, the effect of viability and nonviability on the health benefits in the host must be confirmed for the probiont in question. Methods to evaluate this issue are generally conducted in vivo by comparing the effects of live probiotics to those of inactivated cells, cellular fractions, culture supernatants, and purified compounds (Gatesoupe 2008). Oral administration of live cells was shown to be more effective in pathogen challenge or immune modulation (Brunt and Austin 2005; Panigrahi et al. 2005; Taoka et al. 2006). However, application of freeze-dried probiotics has resulted in disease control (Nikoskelainen et al. 2001a) and immune response (Panigrahi et al. 2007) in rainbow trout, using Lb. rhamnosus and Ent. faecium, respectively. Moreover, Irianto and Austin (2003) demonstrated the potential of dead probiotic cells to control furunculosis in rainbow trout. Salinas et al. (2006) observed an in vitro stimulation of sea bream head-kidney leu-
kocytes by heat-inactivated Lb. delbrueckii. As with live cells, heat-killed Lac. lactis stimulated the immune response of turbot (Scophthalmus maximus) macrophages, but a higher concentration was required compared with live cells (Villamil et al. 2002). Similarly, Villamil et al. (2003a) reported the stimulating effect of nisin, a LAB bacteriocin, toward the phagocytic activity of head kidney macrophages in turbot. Interaction of LAB, their metabolites, or cell wall components and the immunocompetent cells may therefore result in immune modulation in the host. However, inactivation by heat or fixation can cause changes in LAB cell wall composition, affecting the efficacy of the probiont (Panigrahi et al. 2005).

Recently, Merrifield et al. (2011) assessed the effects of vegetative and lyophilized Ped. acidilactici cells on growth, feed utilization, intestinal colonization, and health parameters of rainbow trout by applying two concentrations for 10 weeks. A similar colonization pattern was observed between the two probiont forms; however, increased leukocyte levels were only measured in the live feed at lower concentration. This may suggest that the metabolic activity of freeze-dried probiont was less than that of vegetative cells. In addition, a reduced condition factor was observed in the groups fed the freeze-dried probiont. Still further research is needed in that area to facilitate the application and commercialization of promising probiotics.

28.3.1.5 Administration Vector

Application of probiotics at early stages may induce a desirable larval microbiota and promote survival (Hansen and Olafsen 1989). Probiotic transmission to a host can be achieved by two vectors: diet and rearing water. The administration vector can be a decisive factor on the effects observed. In fact, rearing water treatment will inevitably expose probionts to the three major
routes of infection (skin, gills, and GI tract) available to bacterial adhesion and immune stimulation in the host, while feed supplemented with probiotics will mainly affect the GI tract of the host. Very few studies have compared both vectors during LAB application. Avella et al. (2010) compared the administration of *Lb. rhamnosus* to larval clownfish (*Amphiprion ocellaris*) via live prey with a treatment including both live prey and rearing water supplementation. They observed significantly higher survival, growth, and LAB retention in GI tract, and lower level of skeletal deformities in fish receiving the probiotic via both vectors compared with the live prey vector only. These results were supported by the molecular biomarkers assessed. Nevertheless, both probiotic treatments showed a significant downregulation of the glucocorticoid receptor (stress response biomarker) and led to early fish metamorphosis. The findings may therefore suggest that a higher LAB dose administered via both vectors and resulting GI retention are responsible for the better outcome obtained.

In contrast, Suzer et al. (2008) reported similar beneficial effects in gilthead sea bream (*Sparus aurata*) larvae by supplying multispecies probiotics (intended for pets and swine) either via live prey or both live prey and rearing water, while no improvements were noticed in the rearing water treatment compared with control. Live prey probiotic supplementation was therefore sufficient to increase survival, growth, and activity of pancreatic and intestinal enzymes. Unfortunately, lack of information on probiotic cell concentration used in treatments and levels of probions in the GI tract does not allow for a comprehensive evaluation of this outcome. Taoko et al. (2006) compared the two administration pathways for a multispecies formulation and concluded that the probiotic treatment of rearing water affected the defense system in skin mucus of tilapia (*Oreochromis niloticus*) more than that in plasma, in contrast to oral administration by feeding, which provided an overall better efficacy.

The advantage of probiont addition to rearing water consists in the possibility for early application from the postfertilization stage to modulate the microbiota of ova and eventually larvae before first feeding, since the first possible bacterial accumulation occurs via drinking (Ringø and Vadstein 1998). Indeed, Lauzon et al. (2010a, 2010c) and Strøm and Ringø (1993) reported that early probiotic treatment of ova and/or larvae via rearing water supplementation can reduce bacterial load and modulate their microbiota.

Live prey is an appropriate vector for probiont delivery to fish larvae (Gatesoupe 1994). Bioencapsulation of bacteria in rotifers can be achieved within 20–40 min (Makridis et al. 2000). Villamil et al. (2010) showed that a 1-h treatment of *Ped. acidilactici* supplementation to rotifer culture led to a better probiont retention in rotifers, and alternatively in the gut of fed turbot (*Psetta maxima*) larvae, compared with 24 h. Further, LAB addition to live prey culture may increase their production rate (Gatesoupe 1991; Planas et al. 2004) and modulate their microbiota (Gatesoupe 1991; Villamil et al. 2003b). At a later rearing stage, dry feed is introduced to fish and the advantage of LAB supplementation has been clearly demonstrated by enhanced fish performance, immune response, and disease resistance, as presented in a later section below.

### 28.3.1.6 Dose and Frequency of Application

Maintenance or establishment of probions in rearing systems is considered as a prerequisite for a proof of effects. Frequent LAB administration may contribute to probiont maintenance in the host DT to achieve beneficial effects (Gatesoupe 1999; Nikoskelainen et al. 2003; Panigrahi et al. 2004). Probiont viability may also influence the dose required for beneficial application.

Increased rotifer production over a period of a few days was achieved by a single dose (10⁹–10ⁱ⁰ cfu/ml) of three LAB strains applied to the culture (Planas et al. 2004), while *Vibrio* reduction
Prevalence and Application of Lactic Acid Bacteria in Aquatic Environments

in *Artemia* culture was obtained after a 24-h *Lactobacillus* treatment of $10^8$ cfu/ml (Villamil et al. 2003b). Trials that confirmed the presence of LAB in larvae following live prey supplementation for a few weeks applied a concentration ranging from $10^5$ cfu/ml (live prey culture) to $10^7$ cfu/ml (Gatesoupe 2002; Carnevali et al. 2006; Avella et al. 2010). Even a concentration of $10^8$ cfu/ml (rotifer culture) applied once 3 days post hatch led to the colonization of the probiont in turbot larvae, as confirmed 7 days post treatment (Villamil et al. 2010). Regular LAB application ($10^{6-8}$ cfu/ml) via rearing water has also proved to be beneficial to some larvae and juvenile fish species (Wang et al. 2008; Avella et al. 2010; Lauzon et al. 2010c; Lauzon et al. 2010d), although few trials have been conducted at early stages. Persistence of LAB in larval gut of cod and turbot treated via rearing water was confirmed 7–8 days post treatment at a level of $10^{4-6}$ cfu/g larva (Lauzon et al. 2010c; Lauzon et al. 2010d; Villamil et al. 2010).

Concentration of LAB in reported feed trials ranges from $10^3$ cfu/g to $10^{12}$ cfu/g, with use of higher concentrations generally providing little or no advantage (Nikoskelainen et al. 2001a; Nikoskelainen et al. 2003; Son et al. 2009). Numerous trials applying *Lactobacillus* spp. have been conducted, demonstrating that a concentration as low as $10^3$ cfu/g feed given for 4 weeks induced an immune response in grouper (*Epinephelus coioides*) juveniles, leading to some disease control, while a 100-fold higher concentration led to increased growth and disease resistance (Son et al. 2009). Alternatively, applying a concentration of $10^6$ cfu/g feed for 2 weeks caused an immune response and disease resistance in rainbow trout (Balcazar et al. 2007b). Similar findings have been documented using *Lac. lactis* ssp. *lactis* and *Leuc. mesenteroides* ($10^6$ cfu/g feed) (Balcazar et al. 2007b).

Among reported data, effective concentrations of *Carnobacterium*-supplemented feeds are applied at $10^{-8}$ cfu/g for 2–3 weeks, enhancing immune response and/or disease resistance in rainbow trout, salmon, and cod (Gildberg and Mikkelsen 1998; Robertson et al. 2000; Kim and Austin 2006). Use of *Enterococcus* spp. in feed resulted in increased growth and survival of cod juveniles with $10^7$ cfu/g feed for 55 days (Lauzon et al. 2010b) while a 100-fold higher concentration of another *Enterococcus* strain induced an immune response in rainbow trout (Panigrahi et al. 2007). *Weissella confusa* added to feed ($10^8$ cfu/g) for 30 days enhanced growth and disease resistance in sea bass (Reigpipat et al. 2008). *Ped. acidilactici* feeding ($10^7$ cfu/g) of tilapia for 32 days contributed to increased survival, microbiota control, and immune response (Ferguson et al. 2010). However, a similar treatment applied to rainbow trout for 10 weeks only slightly elicited an immune response (Merrifield et al. 2011).

Probiont persistence in juvenile gut was confirmed post treatment in a few trials, generally until 5–15 days for allochthonous LAB fed rainbow trout, abalone (*Haliotis gigantea*), and grouper (Robertson et al. 2000; Nikoskelainen et al. 2003; Panigrahi et al. 2005; Iehata et al. 2009; Son et al. 2009) in contrast to 3 weeks for autochthonous *Carnobacterium* spp. fed rainbow trout despite a short application (Kim and Austin 2006). In fact, reversion of the immune status was observed after 1 to 2 weeks post treatment by Nikoskelainen et al. (2003) and Panigrahi et al. (2005). This emphasizes the importance of defining autochthonous probionts. It therefore appears that a probiont concentration of $10^5-7$ cfu/g feed given regularly to the host is likely to induce some beneficial effects. However, this can be expected to vary according to the probiont used and the host evaluated. Finally, the use of multispecies probiotics has been suggested to be advantageous, as synergistic effects have been observed (Gatesoupe 2002; Planas et al. 2004; Salinas et al. 2005; Salinas et al. 2008a).

### 28.3.1.7 Safety of Probiotics

The safety assessment of probiotics has been described in several studies, using both *in vitro* and *in vivo* tests to assess their possible interaction with the host. Infectivity of putative probionts to
the host can be easily tested at the larval stage by the multidish method described by Sandlund and Bergh (2008), allowing a rapid screening of several strains at low cost. The ability to produce toxins that induce lysis of erythrocytes has been considered as an important criterion to discard potentially harmful bacteria. Enzymatic properties leading to detrimental effects, such as mucus degradation, and putative virulence factors are undesirable properties (Salminen et al. 1998). Marteau and Shanahan (2003) emphasized the need to further study the risk of translocation and permanent colonization of probionts. Translocation of several pathogens has been described in fish, but not yet with LAB (Birkbeck and Ringø 2005). However, some LAB have been reported in organs of wild and reared aquatic animals (Esteve and Garay 1991; Vela et al. 2008), which could have resulted from translocation.

The integrity of gut mucosa of probiont-treated fish inevitably is a strong criterion to support their safety. More attention should be paid to the effects of probiotics on gut morphology, even when apparently safe strains are being applied. During the last 5 years, several authors have suggested that *Lactobacillus* species isolated from other sources than fish might be good candidates as probiotics in fish (Bagheri et al. 2008; Salinas et al. 2008b; Merrifield et al. 2010b). However, in a recent *ex vivo* study by Salma et al. (2011), it was clearly demonstrated that the probiotic bacterium *Lb. plantarum*, originally isolated from Iranian cheese, caused severe cell damage in the distal intestine of beluga (*Huso huso*) as evaluated by light and electron microscopy. On the basis of their findings, the authors concluded that light and electron microscopy evaluations should be included as an important criterion in future selection of probiotics in fish.

### 28.3.2 LAB Associated with Beneficial Effects Observed

LAB are among several microbial species that have been shown to confer beneficial effects to their host following administration. During the last two decades, there has been great interest in the application of LAB as potential probiotics in aquaculture, as demonstrated by the numerous studies displayed in Tables 28.3 and 28.4. The beneficial effects observed vary according to LAB species used, their origin, the methods of administration (vector, concentration, frequency, or cell viability), the host, and rearing conditions. These important aspects are discussed earlier. However, the mechanisms by which the beneficial effects are achieved have been less examined (Nayak 2010a).

Table 28.3 reveals the wide application of *Lactobacillus* spp. as a probiotic tool in aquaculture, with most of them being of allochthonous nature. *In vivo* trials applying *Carnobacterium* spp. are less numerous but most of the strains used are autochthonous, see Table 28.3. Also, it is noted that research emphasis has been much toward the verification of the effects on the immune response and disease resistance in the host. The data gathered show the wide antagonistic spectrum of *Lactobacillus* spp. against different fish pathogens (*Aer. hydrophila*, *Aer. salmonicida* spp. *salmonicida*, *Lac. garvieae*, *Streptococcus* spp., *Strep. iniae*, *Strep. parauberis*, *Edwardsiella tarda*, and *Pseudomonas fluorescens*) and viruses (lymphocystis disease virus and iridovirus). *Carnobacterium* spp. tested *in vivo* against *Aer. salmonicida*, *Yersinia ruckeri*, *V. anguillarum*, and *V. ordalii* showed promising results, but furunculosis control by *C. divergens* strains was variable (see Table 28.3). Disease control toward *Aer. salmonicida* spp. *salmonicida* was achieved by using *Lac. lactis* spp. *lactis* or *Leuc. mesenteroides*, which was also antagonistic to *Lac. garvieae*. *W. confusa* acted on *Aer. hydrophila*. The improved disease resistance of probionts was generally achieved after a 2- to 4-week feeding period and associated with stimulation of the immune system in the host (Irianto and Austin 2002b; Kim and Austin 2006; Pirarat et al. 2006; Taoka et al. 2006; Balcazar et al. 2007b; Harikrishnan et al. 2010a; Harikrishnan et al. 2010b; Harikrishnan et al. 2010c).
Table 28.3 Application of LAB as Probiotics in Aquaculture by Monospecies Treatments and Beneficial Effects Observed

<table>
<thead>
<tr>
<th>Monospecies</th>
<th>Aquatic Species</th>
<th>Vector</th>
<th>Treatment</th>
<th>Beneficial Effects Observed</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Lactobacillus</strong></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td><em>Lb. casei</em> (Yakult)</td>
<td>all</td>
<td>porthole livebearer (J)</td>
<td>Artemia nauplii, log 6/ml (11 weeks)</td>
<td>SR, no effect on G/S</td>
<td>Hernandez et al. (2010)</td>
</tr>
<tr>
<td><em>Lb. casei</em> ssp. casei CECT4043</td>
<td>all</td>
<td>rotifer</td>
<td>RW</td>
<td>log 9/ml</td>
<td>higher GR, density</td>
</tr>
<tr>
<td><em>Lb. casei</em> ssp. casei CECT4043</td>
<td>all</td>
<td>Artemia nauplii</td>
<td>RW</td>
<td>log 8/ml (24h, 15°C)</td>
<td>Reduced V. alginolyticus in Artemia</td>
</tr>
<tr>
<td><em>Lb. brevis</em> CECT815</td>
<td>all</td>
<td>Artemia nauplii</td>
<td>RW</td>
<td>log 8/ml (24h, 15°C)</td>
<td>Reduced V. alginolyticus in Artemia</td>
</tr>
<tr>
<td><em>Lactobacillus acidophilus</em> (Lactobacil)</td>
<td>all</td>
<td>infecteda carp (J)</td>
<td>feed</td>
<td>log 8/g (4 weeks)</td>
<td>yes, yes</td>
</tr>
<tr>
<td><em>Lactobacillus acidophilus</em> (Lactobacil)</td>
<td>all</td>
<td>infectedb olive flounder (J)</td>
<td>feed</td>
<td>log 8/g (4 weeks)</td>
<td>yes</td>
</tr>
<tr>
<td><em>Lb. paraplantarum</em> all</td>
<td>abalone</td>
<td>feed</td>
<td>log 9–10/g (3 weeks)</td>
<td>yes, 5 d PF: 10^2–3/g</td>
<td>some</td>
</tr>
<tr>
<td><em>Lb. plantarum</em> aut</td>
<td>infectedc rainbow trout (J)</td>
<td>feed</td>
<td>log 6/g (30 d)</td>
<td>none</td>
<td>10^6–7/g digesta</td>
</tr>
<tr>
<td><em>Lb. plantarum</em> all</td>
<td>infectedd grouper (J)</td>
<td>feed</td>
<td>log 3–5–7/g (4 weeks)</td>
<td>G/FE (log 5/g)</td>
<td>yes, 7 d PF: 10^6/g</td>
</tr>
<tr>
<td><em>Lb. rhamnosus</em> IMC 501</td>
<td>all</td>
<td>clownfish (L)</td>
<td>live prey/ LP+RW</td>
<td>log 6/ml, twice daily for 30 d</td>
<td>V/G/D/LD LP+RW &gt;LP</td>
</tr>
</tbody>
</table>

(continued)
Table 28.3  Application of LAB as Probiotics in Aquaculture by Monospecies Treatments and Beneficial Effects Observed (Continued)

<table>
<thead>
<tr>
<th>Monospecies</th>
<th>Aquatic Species</th>
<th>Vector</th>
<th>Treatment</th>
<th>Beneficial Effects Observed</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactobacillus</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Lb. rhamnosus</em> JCM 1136</td>
<td>all</td>
<td>rainbow trout (J)</td>
<td>feed</td>
<td>log 9–11/g (30 d)</td>
<td>yes, in stomach intestine</td>
</tr>
<tr>
<td><em>Lb. rhamnosus</em> JCM 1136</td>
<td>all</td>
<td>rainbow trout (J)</td>
<td>feed LI, FD, IN</td>
<td>log 11/g (30 d)</td>
<td>yes, &lt;15 d PF</td>
</tr>
<tr>
<td><em>Lb. rhamnosus</em> ATCC 53103</td>
<td>all</td>
<td>rainbow trout (J)</td>
<td>feed</td>
<td>log 9/g (45 d)</td>
<td>yes</td>
</tr>
<tr>
<td><em>Lb. rhamnosus</em> ATCC 53103</td>
<td>all</td>
<td>infected* tilapia (J)</td>
<td>feed</td>
<td>log 8–10/g (2 weeks)</td>
<td>yes</td>
</tr>
<tr>
<td><em>Lb. rhamnosus</em> ATCC 53103</td>
<td>all</td>
<td>rainbow trout (J)</td>
<td>feed</td>
<td>log 4–6–8–10–11/g (2 weeks)</td>
<td>yes, &lt;7 or 14 d PF</td>
</tr>
<tr>
<td><em>Lb. sakei</em> BK19</td>
<td>all</td>
<td>infected* kelp grouper (J)</td>
<td>feed (FD)</td>
<td>log 9–12/g (51 d)</td>
<td>yes (log 9/g)</td>
</tr>
<tr>
<td><em>Lb. sakei</em> CLFP202</td>
<td>aut/ all</td>
<td>infected* rainbow trout (J)</td>
<td>feed</td>
<td>log 6/g (2 weeks)</td>
<td>yes, 10^7/g digesta</td>
</tr>
<tr>
<td><em>Lb. sakei</em></td>
<td>aut/ all</td>
<td>brown trout (J)</td>
<td>feed</td>
<td>log 6/g (2 weeks)</td>
<td>yes</td>
</tr>
<tr>
<td><em>Lb. acidophilus</em></td>
<td>all</td>
<td>carp (J)</td>
<td>feed</td>
<td>log 7–8/g (45 d)</td>
<td>G/FE/PE/S</td>
</tr>
<tr>
<td><em>Lb. acidophilus</em></td>
<td>all</td>
<td>infected* tilapia (J)</td>
<td>feed</td>
<td>log 7/g (1–2 mo)</td>
<td>G</td>
</tr>
<tr>
<td><em>Lb. delbrueckii</em> ssp. delbrueckii</td>
<td>aut</td>
<td>sea bream (L)</td>
<td>live prey</td>
<td>log 5/ml (40 days or 59 days)</td>
<td>V/G/D</td>
</tr>
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### Carnobacterium

<table>
<thead>
<tr>
<th>Species</th>
<th>Source</th>
<th>Infection Type</th>
<th>Feed Log</th>
<th>Duration</th>
<th>Pathway</th>
<th>Protein Type</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. malataromaticum</em> B26</td>
<td>aut</td>
<td>infected rainbow trout (J)</td>
<td>log 7/g (14 d)</td>
<td>yes, 3 w PF</td>
<td>yes</td>
<td>cellular, humoral</td>
<td>Kim and Austin (2006)</td>
</tr>
<tr>
<td><em>C. divergens</em> B33</td>
<td>aut</td>
<td>infected rainbow trout (J)</td>
<td>log 7/g (14 d)</td>
<td>yes, 3 w PF</td>
<td>yes</td>
<td>cellular, humoral</td>
<td>Kim and Austin (2006)</td>
</tr>
<tr>
<td><em>C. divergens</em> (LABC)</td>
<td>aut</td>
<td>infected cod (J)</td>
<td>log 8/g (3 weeks)</td>
<td>G</td>
<td>yes, in pyloric ceca $10^3$–$10^4$/g</td>
<td>12 d PI</td>
<td>Gildberg and Mikkelsen (1998)</td>
</tr>
<tr>
<td><em>C. divergens</em> (LABS)</td>
<td>all</td>
<td>infected cod (J)</td>
<td>log 8/g (3 weeks)</td>
<td>G</td>
<td>yes, in PC and intestine $10^2$–$10^3$/g</td>
<td>12 d PI</td>
<td>Gildberg and Mikkelsen (1998)</td>
</tr>
<tr>
<td><em>C. divergens</em></td>
<td>aut</td>
<td>infected cod (J)</td>
<td>log 8/g (3 weeks)</td>
<td>yes</td>
<td>some</td>
<td></td>
<td>Gildberg et al. (1997)</td>
</tr>
<tr>
<td><em>C. divergens</em></td>
<td>aut</td>
<td>infected salmon (J)</td>
<td>log 9/g (5 weeks)</td>
<td>yes, 10$^7$/fish</td>
<td>no</td>
<td></td>
<td>Gildberg et al. (1995)</td>
</tr>
<tr>
<td><em>Carnobacterium</em> sp.</td>
<td>aut</td>
<td>infected Atl salmon (J)</td>
<td>log 7–8/g (7 or 14 d)</td>
<td>yes (14-d)</td>
<td></td>
<td></td>
<td>Robertson et al. (2000)</td>
</tr>
<tr>
<td><em>Carnobacterium</em> sp.</td>
<td>all</td>
<td>infected Atl salmon (J)</td>
<td>log 7–8/g (14 d)</td>
<td>4–10 d PF</td>
<td>yes</td>
<td></td>
<td>Robertson et al. (2000)</td>
</tr>
</tbody>
</table>

### Lactococcus

<table>
<thead>
<tr>
<th>Species</th>
<th>Source</th>
<th>Infection Type</th>
<th>Feed Log</th>
<th>Duration</th>
<th>Pathway</th>
<th>Protein Type</th>
<th>Reference</th>
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<tr>
<td><em>Lac. lactis</em> ssp. lactis CLFP100</td>
<td>aut/ all</td>
<td>infected rainbow trout (J)</td>
<td>log 6/g (2 weeks)</td>
<td>yes, 10$^7$/digesta</td>
<td>yes</td>
<td>yes</td>
<td>Balcazar et al. (2007b)</td>
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<tr>
<td><em>Lac. lactis</em> ssp. lactis</td>
<td>aut/ all</td>
<td>brown trout (J)</td>
<td>log 6/g (2 weeks)</td>
<td>yes</td>
<td></td>
<td>yes</td>
<td>Balcazar et al. (2007a)</td>
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<tr>
<td><em>Lac. lactis</em> ssp. lactis CECT539</td>
<td>all</td>
<td>rotifer RW</td>
<td>log 9/ml</td>
<td>higher GR, density</td>
<td>yes</td>
<td></td>
<td>Planas et al. (2004)</td>
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<tr>
<td><em>Lac. lactis</em> ssp. lactis CECT539</td>
<td>all</td>
<td>turbot (J)</td>
<td>force feeding</td>
<td>log 5/day (7 d)</td>
<td>yes</td>
<td></td>
<td>Villamil et al. (2002)</td>
</tr>
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</table>

(continued)
<table>
<thead>
<tr>
<th>Monospecies</th>
<th>Aquatic Species</th>
<th>Vector</th>
<th>Treatment</th>
<th>Performance</th>
<th>Microbiota Control</th>
<th>Colonization</th>
<th>Enzymatic</th>
<th>Disease Control</th>
<th>Immune Response</th>
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<td><strong>Enterococcus</strong></td>
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<td></td>
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</tr>
<tr>
<td>Ent. thailandicus</td>
<td>all</td>
<td>Atlantic cod (J)</td>
<td>feed</td>
<td>log 7/g (55 d)</td>
<td>S/G/FE</td>
<td>Vibrio effect</td>
<td>low</td>
<td></td>
<td></td>
<td>Lauzon et al. (2010b)</td>
</tr>
<tr>
<td>Ent. mundtii</td>
<td>all</td>
<td>abalone (J)</td>
<td>feed</td>
<td>log 9–10/g (3 weeks)</td>
<td>no</td>
<td>yes</td>
<td></td>
<td></td>
<td></td>
<td>Iehata et al. (2009)</td>
</tr>
<tr>
<td>Ent. faecium (Lactosan GmbH)</td>
<td>all</td>
<td>rainbow trout (J)</td>
<td>feed</td>
<td>log 8/g (10 weeks)</td>
<td>none</td>
<td>yes, 10⁷/g mucosa</td>
<td></td>
<td></td>
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<td>Merrifield et al. (2010a)</td>
</tr>
<tr>
<td>Ent. faecium ZJ4</td>
<td>all</td>
<td>tilapia (J)</td>
<td>RW</td>
<td>log 7–8/ml (every 4 d, for 40 d)</td>
<td>G</td>
<td>yes</td>
<td></td>
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<td>Wang et al. (2008)</td>
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<tr>
<td>Ent. faecium (Japan)</td>
<td>all</td>
<td>rainbow trout (J)</td>
<td>feed (FD)</td>
<td>log 9/g (45 d)</td>
<td></td>
<td>yes</td>
<td></td>
<td></td>
<td></td>
<td>Panigrahi et al. (2007)</td>
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<tr>
<td><strong>Leuconostoc</strong></td>
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<tr>
<td>Leuc. mesenteroides CLFP196</td>
<td>aut</td>
<td>infected rainbow trout (J)</td>
<td>feed</td>
<td>log 6/g (30 d)</td>
<td>none</td>
<td>yes, 10⁶/g digesta</td>
<td>yes</td>
<td></td>
<td></td>
<td>Vendrell et al. (2008)</td>
</tr>
<tr>
<td>Leuc. mesenteroides CLFP196</td>
<td>aut/ all</td>
<td>infected rainbow trout (J)</td>
<td>feed</td>
<td>log 6/g (2 weeks)</td>
<td>none</td>
<td>yes, 10⁷/g digesta</td>
<td>yes</td>
<td>yes</td>
<td></td>
<td>Balcazar et al. (2007b)</td>
</tr>
<tr>
<td>Leuc. mesenteroides</td>
<td>aut/ all</td>
<td>brown trout (J)</td>
<td>feed</td>
<td>log 6/g (2 weeks)</td>
<td>none</td>
<td>yes</td>
<td>yes</td>
<td></td>
<td></td>
<td>Balcazar et al. (2007a)</td>
</tr>
<tr>
<td><strong>Pediococcus</strong></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Ped. acidilactici (Bactocell or MA18/5M)</td>
<td>all</td>
<td>rainbow trout (J)</td>
<td>feed (LI,FD)</td>
<td>log 7–8/g (10 weeks)</td>
<td>none</td>
<td>yes, 10⁷/g mucosa</td>
<td>yes</td>
<td></td>
<td>some (LI)</td>
<td>Merrifield et al. (2011)</td>
</tr>
<tr>
<td>Probiotic Species</td>
<td>Source</td>
<td>Aquatic Animal</td>
<td>Administration</td>
<td>Treatment Duration</td>
<td>Effect</td>
<td>Dosage</td>
<td>References</td>
<td></td>
<td></td>
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</tr>
<tr>
<td><em>Ped. acidilactici</em> (Bactocell)</td>
<td>all</td>
<td>red tilapia</td>
<td>feed</td>
<td>log 7/g (32 d)</td>
<td>S</td>
<td>yes</td>
<td>yes, 10^7–8/g digesta</td>
<td>Ferguson et al. (2010)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Ped. acidilactici</em> MA18/5M</td>
<td>all</td>
<td>rainbow trout (J)</td>
<td>feed</td>
<td>log 6/g (20 d or 5 months)</td>
<td>LD</td>
<td>yes, after 5 months</td>
<td>yes, 10^7–8/g digesta</td>
<td>Aubin et al. (2005)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Ped. acidilactici</em> (Bactocell)</td>
<td>all</td>
<td>pollack (L)</td>
<td>disinfected Artemia</td>
<td>log 7/ml (16 d)</td>
<td>G</td>
<td>yes</td>
<td>10^5/larva</td>
<td>Gatesoupe (2002)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Ped. acidilactici</em> NRRL B-5627</td>
<td>all</td>
<td>turbot (L)</td>
<td>rotifer (1 h/24 h), RW</td>
<td>log 8/ml once 3 dph</td>
<td>none</td>
<td>yes</td>
<td>7 d PT**</td>
<td>Villamil et al. (2010)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Weissella confusa</em></td>
<td>aut</td>
<td>infected o sea bass (J)</td>
<td>feed</td>
<td>log 7/g (30 d)</td>
<td>G</td>
<td>yes</td>
<td></td>
<td>Rengpipat et al. (2008)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Note:**  
J, juvenile; L, larvae; LP, live prey (rotifer and *Artemia*); RW, rearing water; SR, stress resistance; S, survival; V, viability; G, growth; GR, growth rate; FE, feed efficiency; PE, protein efficiency; D, development; LD, less deformities; PF, post feeding; PI, post infection; PT, post treatment; aut or all, autochthonous or allochthonous to the aquatic animal tested; LI, live, vegetative cells; FD, freeze-dried; IN, inactivated; dph, days post hatch.

a Infected by intramuscular (i.m.) injection of *Aer. hydrophila* (9 × 10^4 cfu/fish) 6 days before probiotic feeding treatment.
b Obtained naturally infected by lymphocystis disease virus (LCDV) from a private hatchery.
c Infected by cohabitation (16.7% of fish intraperitoneally (i.p.) injected with *Lac. garvieae* CLFP LG1, 3.4 × 10^8 cells/fish) after 30-day feeding treatment.
d Infected i.m. by *Streptococcus* sp. (5.6 × 10^7 cfu/g fish) or i.p. by a grouper iridovirus (1.7 × 10^7 TCID50/g fish) after 4-week feeding treatment.
e Infected i.p. by *Edwardsiella tarda* E381 (log 9 cfu/fish) after 2-week feeding treatment.
f Infected by cohabitation (15% of fish i.p. injected with *Aer. salmonicida* ssp. *salmonicida*, log 6 cfu/fish) after 16-day feeding treatment.
g Infected i.p. at day 0 with *Strep. iniae* or *Strep. pararuberis* separately or mixed (~log 6 cells/fish).
h Infected by cohabitation (15% of fish i.p. injected with *Aer. salmonicida* ssp. *salmonicida* CLFP501, 1.7 × 10^6 cells/fish) after 2-week feeding treatment.
i Infected i.p. by *Aer. hydrophila*, *Pseudomonas fluorescens* and *Strep. iniae* (log 8/fish) after 1 or 2 months feeding treatment.
j Infected i.p. by *Aer. salmonicida* Hooke (2.4 × 10^6 cells/fish) and *Yersinia ruckeri* T1 (1.6 × 10^6 cells/fish) after 14-day feeding treatment.
k Infected by batch challenge with *V. anguillarum* LFI 1243 (log 7/ml rearing water for 1 h) after 3-week feeding treatment.
l Infected by cohabitation (5% of fish i.p. injected with *Aer. salmonicida* AL 2020; 5.8 × 10^6 cells/fish) after 5-week feeding treatment.
m Infected by cohabitation (16.7% of fish i.p. injected with *Aer. salmonicida* Hooke, *Y. ruckeri* PR110 or *V. ordalii* V453, log 6 cfu/fish) after 7-day and 14-day feeding treatment.  
   Challenging with *V. anguillarum* V72 could not be mediated by the probiont used.

Infected by cohabitation (16.7% of fish i.p. injected with *Aer. salmonicida* Hooke or *Y. ruckeri* PR110, log 6 cfu/fish) after 14-day feeding treatment.

Infected by immersion in aquaria containing *Aer. hydrophila* (log 7/ml) after 30-day feeding treatment.

* Lb. rhamnosus IMC 501 levels in disinfected whole larvae reached log 7–8 cfu/g larva fed live prey, but 10-fold higher levels in combined treatment.

** Levels of *Ped. acidilactici* in larvae 7 days post treatment varied according to administration pathways: log 5 cfu/larva via rearing water, log 2–3 cfu/larva via 1-h-treated rotifers, but log 1–2 cfu/larva via 24-h-treated rotifers.
<table>
<thead>
<tr>
<th>Multispecies</th>
<th>Aquatic Species</th>
<th>Vector</th>
<th>Treatment</th>
<th>Beneficial Effects Observed</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. divergens V41 + Ent. thailandicus + Arthrobacter bergerei</td>
<td>all/aut</td>
<td>Atlantic cod (O/L)</td>
<td>RW</td>
<td>log 6–7/ml (3 times pf + 5 times ph)</td>
<td>survival, viability, growth</td>
</tr>
<tr>
<td>Ent. thailandicus + Art. bergerei</td>
<td>aut</td>
<td>Atlantic cod (O/L)</td>
<td>RW</td>
<td>log 6/ml (2 times pf + 6 times ph)</td>
<td>growth, development</td>
</tr>
<tr>
<td>Lb. delbrueckii ssp. lactis + Bacillus subtilis</td>
<td>all</td>
<td>sea bream (J)</td>
<td>feed*</td>
<td>log 7/g (3 weeks) IN by heat</td>
<td>survival, growth</td>
</tr>
<tr>
<td>Lb. fructivorans + Lb. plantarum</td>
<td>aut all</td>
<td>sea bream (L)</td>
<td>LP/feed Art./feed</td>
<td>log 5/ml From d5 to 99 From d27 to 99</td>
<td>none</td>
</tr>
<tr>
<td>Lb. farcininis + Lb. rhamnosus (SORBIAL)</td>
<td>all</td>
<td>sea bass (J)</td>
<td>feed</td>
<td>log 8 to 9/g (103 d) L1 or IN** S/D/LD</td>
<td>yes</td>
</tr>
<tr>
<td><strong>Lb. acidophilus</strong> + <strong>Ent. faecium</strong> + <strong>Bifidobacterium bifidum</strong></td>
<td>bullfrog</td>
<td>feed</td>
<td>log 5/g (112 d)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
</tbody>
</table>
| **Probiotics**  
**Acidophilus** | all | cutthroat trout (J) | feed | log 9/g (FD) (71 d) | G/FE |  | (Arndt and Wagner 2007) |
| **Carnobacterium sp., Aer. hydrophila, V. fluvialis, G+ cocci** | aut | rainbow trout, infected (J) | feed | log 7/g (7 or 14 d) | none | yes, 7 d PF | yes yes (Irianto and Austin 2002b) |
| Four pre-sumptive LAB strains (cocci) | aut | tilapia (J) in suboptimal environment | feed | log 4/g (136 d) | growth |  | (Apun-Molina et al. 2009) |
| **Lb. acidophilus** + **B. subtilis** ATCC 6633 | all | tilapia (J), infected b | feed* | log 7/g (1 or 2 mo) | survival | yes yes | (Aly et al. 2008) |
| **Lb. acidophilus** + **B. subtilis** + **Cl. butyricum** + **S. cerevisiae** | all | tilapia, infected c | feed (LI/IN) or RW (LI) (30 d) *** | log 7/g daily RW: log 4 to 5/ml every 5 days | V |  | (Taoka et al. 2006) |

(continued)
Table 28.4 Application of LAB as Probiotics in Aquaculture by Multispecies Treatments and Beneficial Effects Observed (Continued)

<table>
<thead>
<tr>
<th>Multispecies</th>
<th>Aquatic Species</th>
<th>Vector</th>
<th>Treatment</th>
<th>Beneficial Effects Observed</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lb. casei ssp. casei + Lac. lactis ssp. lactis + Ped. acidilactici</td>
<td>all rotifer</td>
<td>RW</td>
<td>log 9–10/ml</td>
<td>S/GR</td>
<td>(Planas et al. 2004)</td>
</tr>
</tbody>
</table>

Note: J, juvenile; L, larvae; O, ova; LP, live prey (rotifer and Artemia); RW, rearing water; S, survival; V, viability; G, growth; GR, growth rate, FE, feed efficiency; D, development; PF, post feeding; PT, post treatment; pf, post fertilized; ph, post hatch; aut or all, autochthonous or allochthonous to the aquatic animal tested; L, live, vegetative cells; FD, freeze-dried; IN, inactivated.

a Infected by Aer. salmonicida after 14-day feeding treatment either by immersion (2 g fish in log 7 cfu/ml for 5 min) or cohabitation (20% of 12-g fish i.p. injected with log 6 cfu/fish).
b Infected i.p. by Aer. hydrophila, P. fluorescens and Strep. iniae (log 8 cfu/fish) after 1 or 2 months feeding treatment.
c Infected i.p. by Edw. tarda (5 × 10^6 cfu/fish) after 30-day feeding or rearing water treatment.

* Generally greater beneficial effects observed when applying a multispecies treatment compared with monospecies treatments.
** No significant difference among beneficial effects achieved with live and inactivated bacterial cells.
*** Best outcome with live cells added to feed.
† Presumptive identification of strains.
§ Contains Lb. plantarum, Lb. rhamnosus, Lb. casei, Lb. paracasei, Lb. salivarius, Bifidobacterium bifidum, and B. longum.
Enhancement of fish performance (survival, growth, viability, feed utilization, development, and less deformities) by application of probiotics has also been widely documented (Farzanfar 2006; Nayak 2010a). It is an important attribute since better quality juvenile may result, ensuring a sustainable production. Several Lactobacillus spp., Enterococcus spp., Ped. acidilactici, and C. divergens have been reported to promote better fish performance as indicated in Tables 28.3 and 28.4. Apparent colonization or even persistence of LAB in the gut of probiont-treated fish has been verified by some workers, but differences in methodology do not always allow for the confirmation of true colonization of the gut mucosa. Based on Tables 28.3 and 28.4, less emphasis has been placed toward the assessment of the influence of LAB probionts on the host’s GI enzymatic activity and microbiota, but positive results have been documented (Villamil et al. 2003b; Aubin et al. 2005; Frouel et al. 2008; Iehata et al. 2009; Ferguson et al. 2010; Lauzon et al. 2010b; Lauzon et al. 2010c; Lauzon et al. 2010d).

Finally, the data presented indicate the lack of studies dealing with early stages of life where larval survival and malformation problems occur. Early application of probiotics has been shown to provide a better enhancement of beneficial effects (Carnevali et al. 2006; Picchietti et al. 2007). Further, few studies have considered the effect of long-term probiont application and the optimum duration of a probiotic treatment.

### 28.4 Conclusions and Future Perspectives

Interest in LAB from aquatic environments is growing as demonstrated by recent publications. Better isolation methods and developments in molecular techniques have contributed to a better understanding of aquatic microbial ecology. During the last two decades, several comprehensive review papers have been published focusing on the use of probiotics in aquaculture related to growth, improving immune activity by improving barrier properties of mucosa, modulating production of cytokines, modulating the gut microbiota, improvement of fish diseases, competition between the “good” and pathogenic bacteria in the fish gut, and production of antimicrobial compounds (Ringø and Birkbeck 1999; Verschuere et al. 2000; Irianto and Austin 2002a; Gram and Ringø 2005; Ringø et al. 2005; Balcazar et al. 2006; Gatesoupe 2007; Panigrahi and Azad 2007; Magnadottir 2010; Merrifield et al. 2010b; Nayak 2010a; Ringø et al. 2010a). However, only one recent study (Askarian et al. 2011b) has evaluated enzyme-producing bacteria combined with in vitro growth inhibition of pathogenic bacteria. Such beneficial effect combined with the ability to detoxify antinutrients merits further investigations as inclusion of vegetable-based products, which are becoming usual in commercial aquaculture because of shortage of fish meal and fish oil.

### References


Prevalence and Application of Lactic Acid Bacteria in Aquatic Environments


Lactic Acid Bacteria: Microbiological and Functional Aspects


Prevalence and Application of Lactic Acid Bacteria in Aquatic Environments


Prevalence and Application of Lactic Acid Bacteria in Aquatic Environments


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Chapter 29

Probiotics for Farm Animals

Alojz Bomba, Radomíra Nemcová, Ladislav Strojný, and Dagmar Mudroňová

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29.1 Introduction

The colonization of the digestive tract in animals begins soon after birth, and the normal microbiota changes dramatically during the life of the host. The composition of the gastrointestinal microbiota differs between animal species. In principle, the role of gut microbiota in animals is similar to those in humans but some of its functions are emphasized in farm animals with regard to their environment, character of their feed, and the economy of farm animal rearing. The composition and metabolism of the gastrointestinal microbiota affects the performance of farm animals in many ways, especially in the young, which are subjected to many stressful conditions. With progression of age, the composition of the feed changes and the morphology and function of the gastrointestinal tract develops. With this, certain differences gradually occur in the composition of the microbiota in calves, lambs, suckling piglets, and chicks that are typical for the given animal species. The gut ecosystem of adult animals is stable and changes only due to the effects of external factors of an adequate intensity (long-lasting change of feed, stress, administration of antibiotics). In farm animals, the microbiota of the digestive tract plays an important role both in the process of optimal development and growth of the organism, as well as in securing the resistance of the animal to diseases. However, due to various adverse impacts, disturbances in optimal growth, production and health state of the animals are rather frequent in animal production (Bomba et al. 2006a).

To ensure optimal growth, production, and health of farm animals, the beneficial microbiota of the gastrointestinal ecosystem can be supported by manipulation of the diet and application of probiotic microorganisms. Probiotics could represent an effective and safe alternative to the use of synthetic substances, for example, antibiotics, in nutrition and medicine (Bomba et al. 2006a; Bomba et al. 2006b). The Food and Agriculture Organization (FAO) and World Health Organization (WHO) (2001) defined probiotics as “live microorganisms which when administered in adequate amounts confer a health benefit on the host.” The particularities of the use of probiotics in farm animals consist in their application not only for disease prevention but also for optimization of animal production. Probiotics may be effectively used particularly in modulation of the gastrointestinal ecosystem, digestive processes, growth stimulation, and in the prevention and therapy of digestive tract diseases in young farm animals.

*Lactobacillus bulgaricus*, *L. acidophilus*, *L. casei*, *L. helveticus*, *L. lactis*, *L. salivarius*, *L. plantarum*, *Streptococcus thermophilus*, *Enterococcus faecium*, *E. faecalis*, *Bifidobacterium* spp., and particular strains of *Escherichia coli* are most frequently used for probiotic purposes. All the above-mentioned microorganisms, except for *L. bulgaricus* and *S. thermophilus*, which are starter cultures of yogurt, form natural components of the gut microbiota (Fuller 1989) and are used as veterinary probiotics. Also *Bacillus* spp. are used as probiotics for veterinary applications, but fall outside the scope of this book. From the viewpoint of the practical use of probiotics, it is of particular importance that probiotics have both local and general biomedical effects, such as inhibitory effect against pathogens, optimizing effects on digestive processes, and immuno-stimulating effects.
29.2 Probiotics for Pigs

The first week of life and weaning can be considered as two crucial periods in pig breeding. The period immediately after birth is probably the most critical one in the whole life of a pig. In this period significant growth, morphological changes, and maturation of the gastrointestinal tract take place. The microbial colonization of the porcine intestine begins at birth and follows a rapid succession during the neonatal and weaning period. Following the withdrawal of sow’s milk, the piglets are highly susceptible to enteric diseases partly as a result of the altered balance between developing beneficial microbiota and the establishment of intestinal bacterial pathogens. The intestinal immune system of the newborn piglet is poorly developed at birth and undergoes a rapid period of expansion and specialization that is not completed before early weaning (Lallés et al. 2007). It is very important to modulate the gastrointestinal microbiota of piglets at an early age because in this period, diarrheal diseases with high morbidity and mortality rates present an extraordinary serious health and economic problem.

The period of weaning is stressful because piglets 3–4 weeks of age are separated from the mother and mixed with piglets from other litters. At weaning, there is a drastic change of diet to dry feed. It may cause dysfunction of the intestinal barrier and lead to indigestion and malabsorption during weaning, predisposing piglets to enteric infections (Chin and Chapman 2009). Consequently, a decrease in daily weight gains brings along risk for growth retardation. Gastrointestinal disturbances include alterations in small intestine architecture and enzyme activities. Recent data indicate a transient-increased mucosal permeability, disturbed absorptive-secretory electrolyte balance, and altered local inflammatory cytokine pattern after weaning (Lallés et al. 2007).

29.2.1 Health Benefits and Application

In pigs, probiotics can be effectively used in neonatal piglets and during the period of weaning. Probiotics in neonatal piglets can be used to support the development of a stable microbiota, to stimulate the immune system and to prevent diarrheal diseases. During the weaning and post-weaning period, probiotics in pigs are used to prevent postweaning diarrhea (PWD) and stimulation of growth.

29.2.1.1 Use of Probiotics in Modulation of Gastrointestinal Ecosystem and Diarrheal Disease Prevention in Neonatal Piglets

The gastrointestinal microbiota of young piglets is composed of facultative anaerobic microorganisms in the proximal intestine (duodenum, jejunum) whose number ranges from $10^3$ to $10^7$ per gram of content (Ducluzeau 1985). This number increases progressively in the ileum, and in the last parts of the digestive tract strictly anaerobic bacteria comprise the dominant microbiota. In very young piglets, *E. coli* is the dominant microbe of all gut segments, together with species of the genera *Lactobacillus* and *Streptococcus*. The microbiota of the piglet progressively changes with age, the numbers of *E. coli* decrease in all segments, and the lactobacilli and streptococci constitute the dominant microbiota of the proximal intestine. The presence of lactobacilli as a constituent of the normal microbiota of the gastrointestinal tract is considered to be beneficial to the porcine host (Tannock 1990). The strictly anaerobic microbiota becomes more diversified in the distal segments, where *Bacteroides*, *Eubacterium*, *Peptostreptococcus*, and many *Clostridium* species are found (Ducluzeau 1985).
Gastrointestinal diseases are often the major cause of morbidity and mortality in piglets at an early stage of life. Enterotoxigenic *E. coli* are frequently identified as causative agents of diarrheal diseases of infectious etiology in neonatal and weaned pigs (Fairbrother 1993; Moon and Bunn 1993). The pathogenicity of *E. coli* is conditional on two factors: the presence of colonization factors enabling the carrier to colonize the mucosa of the small intestine and the ability to produce enterotoxin (Smith and Gyles 1970; Jones and Rutter 1972). The ability of enterotoxigenic *E. coli* to colonize the gut presents the primary and decisive pathogenic factor since it is inevitable for the second virulence factor—to exert its effect. For this reason, effective probiotic should be able to prevent the adhesion of pathogens to intestinal mucosa.

*L. fermentum*, *L. acidophilus*, *L. reuteri*, *L. plantarum*, *L. bulgaricus*, *L. casei*, *Enterococcus faecium*, and *E. faecalis* are used for probiotic purposes in pigs (Lim and Tan 2009). Probiotics in neonatal piglets can be effectively used to support the development of a stable microbiota and to prevent diarrheal diseases. Probiotics as natural bioregulators help maintain the balance of the digestive tract ecosystem by a variety of mechanisms and prevent colonization of the digestive tract by pathogenic bacteria (Fourniat et al. 1992; Vandenbergh 1993; Ávila et al. 1995). The mode of inhibitory action of probiotics against pathogens may be mediated by competition for receptors on the gut mucosa, competition for nutrients (Freter 1992), the production of antibacterial substances (Piard and Desmazeaud 1991), and stimulation of the immune system (Perdigón and Alvarez 1992). Blomberg et al. (1993) observed *L. fermentum* 104R produced a proteinaceous component detectable in spent culture fluid during growth in both complex and defined media; this component inhibited adhesion of K88ab and K88ac fimbriae to ileal mucus by interacting with mucus components. Muralidhara et al. (1973) observed that administration of lactobacilli fully inhibited the adherence of enterotoxigenic *E. coli* in the first three segments of the small intestine that had been divided into nine segments. The antienterotoxic activity of lactobacilli was reported by Mitchel and Kenworthy (1976).

The data concerning the efficacy of probiotics in the prevention of diarrheal diseases in young animals are contradictory. The effect of lactobacilli and bifidobacteria against diarrhea in pigs was confirmed by several reports (Maeng et al. 1989; Depta et al. 1998). However, other authors (De Cupere et al. 1992; Bekaert et al. 1996) have not confirmed this effect. These contradictory results can be explained by the low doses of the used species of probiotic bacteria, strain difference between probiotics, their stability, interactions with other medications, and the health condition, age, and genetic predisposition of the animals (Kyriakis et al. 1999).

Probiotics are most effective in animals during microbiota development or when microbiota stability is impaired (Stavric and Kornegay 1995). To obtain the highest effect of probiotic preparations, supplementation of piglets shortly after birth is recommended (Vondrusková et al. 2010). Feeding of probiotics to sows before farrowing and during lactation and to neonatal piglets decreased the numbers of pathogenic microorganisms in sow and piglet feces, and resulted in the reduced occurrence of digestive disorders and mortality (Danek et al. 1991; Martin et al. 2009).

Using a gnotobiotic pig model inoculated with *E. coli* O8: K88 at the age of 5 days, it was shown that continuous application (from day 2 to day 10 of age) of *L. casei* was more effective in comparison with short-term application (from day 2 to day 4 of age). Continuous application of *L. casei* significantly increased the percentage of phagocytic activity (%PA) at 7 days of age (*p* < .05) and significantly decreased the number of *E. coli* colonizing the jejunum of pigs at 10 days of age (*p* < .05) in comparison with the control group, while the short-term application of lactobacilli did not affect the *E. coli* colonization (Herich et al. 1999; Bomba et al. 1998).

Despite substantial knowledge obtained, the mode of action of probiotics has not been fully explained yet. To enhance the efficacy of probiotics, it is necessary to obtain additional knowledge...
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on the mechanisms underlying their efficacy in the digestive tract (Stavric and Korgenay 1995). If probiotics are to represent a real and effective alternative to antibiotics, it is absolutely necessary to ensure their consistent high effectiveness. The efficacy of probiotics may be potentiated by several methods: the selection of more efficient strains, genetic manipulation, the combination of several strains, and the combination of probiotics and synergistically acting components of a natural origin.

Synbiotics are the combination of both probiotics and prebiotics, which stimulate the growth and/or the activities of both exogenous (probiotic) and endogenous bacteria. Synbiotics seem to be preparations whose potentiated protective and stimulating effects occur in the colon. In various disorders of the gastrointestinal tract, there occur conditions for the translocation of pathogens from the colon into the proximal part of the digestive tract. Taking into account the pathogenesis of some diseases, there is a need to protect the digestive tract mucosa throughout its length, including the small intestine, so that the adhesion of pathogenic microorganisms can be prevented. Potentiated probiotics may fulfill such function. Potentiated probiotics are defined as biopreparations containing strains of microorganisms and synergistically acting components of natural origin that potentiate the probiotic effect on both the small intestine and the colon and their beneficial effect on the host by intensifying a mechanism or by extending the range of their probiotic action (Bomba et al. 2002a; Bomba et al. 2002b; Bomba et al. 2006a). It seems that to potentiate the effect of probiotics, a number of suitable components may be used such as oligosaccharides, maltodextrin, plants and their extracts, and poly-unsaturated fatty acids. The preventive administration of *L. casei* alone during the first week of life resulted in only a slight inhibitory effect on the adhesion of *E. coli* to jejunal mucosa, while *L. casei* administered in combination with maltodextrin decreased the number of *E. coli* colonizing jejunal mucosa by 1 log in gnotobiotic pigs and by more than 2.5 log in conventional pigs at the age of 7 days (Bomba et al. 1999). Nemcová et al. (2007) showed that the combination of *L. plantarum*, maltodextrin and oligosaccharides (fructooligosaccharides [FOS]) proved to be the most effective one to inhibit the counts of *E. coli* O8:K88 adhering to the intestinal mucosa of the jejunum and colon of conventional piglets at the age of 7 days in comparison with a combination of *L. plantarum* and FOS, a combination of *L. plantarum* and maltodextrin, and *L. plantarum* applied alone (*p < .05). Table 29.1 shows the used probiotic strains of microorganisms and their beneficial effects in pigs.

29.2.1.2 Use of Probiotics in PWD Prevention and Immune Modulation in Pigs during the Weaning and Postweaning Period

The weaning time is a crucial period in the management of piglets. A change of the gut environment occurs in connection to weaning of the piglets. Weaning and weaning age have significant effects on microbial population and volatile fatty acids (VFA) concentration (Franklin et al. 2002). Early weaned piglets are exposed to different stress factors associated with changes of nutrition, development of the gastrointestinal tract and immune system. Associated with weaning are marked changes in histology and biochemistry of the small intestine, such as villous atrophy and crypt hyperplasia, which caused decreased digestive and absorptive capacity (Pluske et al. 1997) and contribute to PWD. The major factors implicated in the etiology of these changes are change in nutrition, stress due to separation from mother and littermates, new environment, the withdrawal of milk-borne growth-promoting factors, as well as enteropathogens and their interactions with the gut microbiota. Weaning stress activates the enteric neuronal network in pigs that is accompanied by coactivation of prostanoid pathway. This is associated with increased expression of intestinal corticotropin-releasing factor receptors that trigger mucosal dysfunctions such
Table 29.1 Effect of LAB in Pigs

<table>
<thead>
<tr>
<th>Microorganism Used as Probiotic</th>
<th>Age and Beneficial Effects</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Lactobacillus casei</em> CCM4160</td>
<td>Reduced occurrence of diarrheal syndrome in neonatal piglets</td>
<td>Danek et al. (1991)</td>
</tr>
<tr>
<td><em>Lactobacillus casei</em></td>
<td>Significant increase in the percentage of phagocytic activity (%PA) at 7 days of age <em>(p &lt; .05)</em>, and significant decrease in the number of <em>E. coli</em> O8: K88 colonizing the jejunum of pigs at 10 days of age</td>
<td>Herich et al. (1999)  Bomba et al. (1998)</td>
</tr>
<tr>
<td><em>Lactobacillus casei</em> in combination with maltodextrin</td>
<td>Significant decrease in the number of <em>E. coli</em> O8: K88 colonizing the jejunum of pigs at 7 days of age</td>
<td>Bomba et al. (1999)</td>
</tr>
<tr>
<td><em>Lactobacillus plantarum</em> in combination with maltodextrin and fructooligosaccharides</td>
<td>Significant decrease in the counts of <em>E. coli</em> O8:K88 adhering to the intestinal mucosa of the jejunum and colon of piglets at the age of 7 days</td>
<td>Nemcová et al. (2007)</td>
</tr>
<tr>
<td><em>Lactobacillus rhamnosus</em> GG</td>
<td>Ameliorated diarrhea in postweaning piglets, induced by <em>E. coli</em> K88; significantly increased secretory IgA and TNF-α concentrations; and attenuated the elevation of serum IL-6 induced by <em>E. coli</em></td>
<td>Zhang et al. (2010)</td>
</tr>
<tr>
<td><em>Lactobacillus fermentum</em> I5007</td>
<td>Enhanced T-cell differentiation and induced ileum cytokine expression in weaned piglets</td>
<td>Wang et al. (2009)</td>
</tr>
<tr>
<td><em>Enterococcus faecium</em> SF68</td>
<td>Decline in the frequency of β-hemolytic and O141 serovars of <em>E. coli</em> in pigs at the age of 8 weeks</td>
<td>Scharek et al. (2005)</td>
</tr>
<tr>
<td><em>Enterococcus faecium</em> NCIMB 10415</td>
<td>Greater production of specific antibodies (serum IgM and IgA levels) against <em>Salmonella</em> serovar Typhimurium DT104 in weaning piglets</td>
<td>Szabó et al. (2009)</td>
</tr>
<tr>
<td><em>Lactobacillus paracasei</em> alone and mixture of <em>L. paracasei</em> and fructooligosaccharides</td>
<td>Significantly increased <em>Lactobacillus</em>, <em>Bifidobacterium</em>, total anaerobes and aerobes counts; significantly decreased <em>Clostridium</em> and <em>Enterobacteriaceae</em> counts in the feces; and significantly increased total counts of CD4+ T cells and B cells in weaning piglets</td>
<td>Nemcová et al. (1999)  Herich et al. (2002)</td>
</tr>
<tr>
<td><em>Lactobacillus reuteri</em> BSA131</td>
<td>Beneficial effect on average weight gain, feed conversion, and a decrease in the number of enterobacteria in the feces of 1-month-old piglets</td>
<td>Chang et al. (2001)</td>
</tr>
</tbody>
</table>

(continued)
as a reduction in tight junction function (measured as transepithelial electrical resistance) and increased intestinal permeability (Moeser et al. 2007). The short period after weaning is characterized by a lower food intake and generally by an energy-deficient state (Fenton et al. 1985). During the first week after weaning, pH and the content of dry matter decrease, as well as the count of lactobacilli, while the number of coliform bacteria increases (Jensen 1998). These changes contribute to low weight gains and predisposition to diarrhea.

PWD is one of the most frequent causes of heavy economic losses in pig herds. PWD can be caused by a number of causative agents. Enterotoxigenic *E. coli* strains are generally considered to be the main cause of diarrhea at weaning and the period immediately thereafter. PWD can be also caused by members of the genera *Clostridium*, *Lawsonia*, and *Brachyspira* (Vondrusková et al. 2010). Rotaviruses, corona viruses, and transmissive gastroenteritis virus are frequently identified as causative viral agents (Song et al. 2006; Thomsson et al. 2008).

Weaning considerably influences the health state of piglets; hence it is important to stimulate the indigenous intestinal microbiota and to maintain the balance of the digestive tract microbiota because it counteracts the colonization of the gastrointestinal tract by pathogens (Jensen 1998). Antibiotic growth promoters were used to prevent the PWD, but their use was banned in European Union by 2006. There is a need to develop the effective alternatives. The use of probiotics represents an efficacious strategy in PWD prevention.

Probiotic application to piglets is important especially in the postweaning period for the prophylaxis of PWD, which is usually caused by enterotoxigenic *E. coli* strains (Bomba et al. 2002a; Marinho et al. 2007). Probiotics containing different *Lactobacillus* species beneficially affected the occurrence of diarrheal diseases in weanling pigs (Mathew et al. 1998; Scheuermann 1993). *Lactobacillus rhamnosus* GG (LGG) was effective in ameliorating diarrhea in postweaning

### Table 29.1 Effect of LAB in Pigs (Continued)

<table>
<thead>
<tr>
<th>Microorganism Used as Probiotic</th>
<th>Age and Beneficial Effects</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Lactobacillus acidophilus</em></td>
<td>Tendency to improve average weight gain and feed conversion in 4-week-old piglets</td>
<td>Pollmann et al. (1980b)</td>
</tr>
<tr>
<td><em>Lactobacillus</em> sp.</td>
<td>Increased feed intake and weight gain in weaned pigs</td>
<td>Lessard and Brisson (1987)</td>
</tr>
<tr>
<td><em>Bifidobacterium lactis</em> HN019</td>
<td>Reduced weaning diarrhea associated with rotavirus and <em>E. coli</em> infection, and beneficial effect on performance of piglets</td>
<td>Shu et al. (2001)</td>
</tr>
<tr>
<td><em>Enterococcus faecium</em> NCIMB 10415</td>
<td>Significantly increased serum IgG (<em>p</em> &lt; .05) in weaned piglets</td>
<td>Scharek et al. (2007b)</td>
</tr>
<tr>
<td><em>Bacillus cereus</em> var. toyoi</td>
<td>Significantly enhanced population of intraepithelial of CD8+ T cells (<em>p</em> ≤ .05), the numbers of γδ T cells tended to be higher in the intestinal epithelium (<em>p</em> &lt; .01) at the time of weaning (day 28), significantly more CD25+ lymphocytes and γδ T cells in the probiotic group postweaning, less frequent occurrence of pathogenic <em>E. coli</em> serogroups in the feces</td>
<td>Scharek et al. (2007a)</td>
</tr>
</tbody>
</table>
piglets induced by \textit{E. coli} K88, possibly via modulation of intestinal microbiota, enhancement of intestinal antibody defense, and regulation of production of systemic inflammatory cytokines. Administration of LGG significantly increased secretory IgA and TNF-\(\alpha\) concentrations and attenuated the elevation of serum IL-6 induced by \textit{E. coli} (Zhang et al. 2010). Wang et al. (2009) reported that oral administration of \textit{L. fermentum} I5007 can enhance T-cell differentiation and induce ileum cytokine expression, suggesting that this probiotic strain could modulate immune function in weaned piglets. The administration of \textit{L. reuteri} BSA131 decreased the number of enterobacteria in the feces (Chang et al. 2001).

At an age of 8 weeks, no differences were observed in the populations of CD4+ and CD8+ T cells in Peyer’s patches in pigs administered with probiotic \textit{E. faecium} SF68 strain. However, the levels of cytotoxic T cells (CD8+) in the jejunal epithelium of piglets of the probiotic group were significantly reduced. The depth of the jejunal crypts and length of the villi were similar in both groups, suggesting the relative T-cell population differences were not due to alterations in the epithelial cell numbers. The total anaerobe and coliform bacterial populations were not significantly affected by the probiotic treatments. However, a remarkable decline in the frequency of \(\beta\)-hemolytic and O141 serovars of \textit{E. coli} was observed in the intestinal contents of probiotic piglets, suggesting an explanation for the reduction in cytotoxic T-cell populations (Scharek et al. 2005). Significantly increased serum IgG after application of \textit{E. faecium} NCIMB 10415 have been observed in weaned piglets (Scharek et al. 2007b). \textit{E. faecium} NCIMB 10415 treatment enhanced the course of infection in weaning piglets challenged with \textit{Salmonella} serovar Typhimurium DT104. However, the probiotic treatment also appeared to result in greater production of specific antibodies (serum IgM and IgA levels) against \textit{Salmonella} serovar Typhimurium DT104 (Szabó et al. 2009).

Shu et al. (2001) showed that probiotic treatment using \textit{Bifidobacterium lactis} HN019 reduced weaning diarrhea associated with rotavirus and \textit{E. coli} infection in pigs. Lessard et al. (2009) indicated that \textit{Pediococcus acidilactici} and \textit{Saccharomyces cerevisiae} var. \textit{boulardii} may have the potential to modulate establishment of lymphocyte populations and IgA secretion in the gut and to reduce bacterial translocation to mesenteric lymph node after enterotoxigenic \textit{E. coli} infection. In a feeding trial, sows and piglets were fed with the probiotic bacterium \textit{Bacillus cereus} var. \textit{toyoi} as a feed additive. The population of intraepithelial CD8+ T cells was significantly enhanced in the probiotic group piglets, and the numbers of \(\gamma\delta\) T cells tended to be higher in the intestinal epithelium at the time of weaning (day 28). Lamina propria lymphocytes were also influenced by the treatment. Application of \textit{B. cereus} var. \textit{toyoi} resulted in significantly more CD25+ lymphocytes and \(\gamma\delta\) T cells in the probiotic group postweaning. The occurrence of pathogenic \textit{E. coli} serogroups was also less frequent in the feces of piglets from the probiotic group. The finding that the CD8+ T-cell population in the intestinal mucosa showed changes on day 28, indicating an influence of \textit{B. cereus} var. \textit{toyoi} supplementation on the intestinal immune system before weaning, an observation supported by changes in the intestinal microbiota observed during the suckling period. The results suggest that feeding of \textit{B. cereus} var. \textit{toyoi} to sows may result in beneficial effects on piglet health status independent of their feed supplementation (Scharek et al. 2007a).

Probiotics may represent an attractive alternative to antibiotics and current research should be aimed at improving of their efficacy. Nemcová et al. (1999) and Herich et al. (2002) investigated the influence of administration of \textit{L. paracasei} alone and mixture of \textit{L. paracasei} and FOS on fecal bacteria counts in the weanling pigs. Administration of \textit{L. paracasei} alone significantly decreased \textit{Clostridium} and \textit{Enterobacteriacea} counts as compared with the control. \textit{L. paracasei} administered in combination with FOS significantly increased \textit{Lactobacillus}, \textit{Bifidobacterium}, total aerobes, and total anaerobes counts compared with the control group as well as \textit{L. paracasei} group and significantly decreased \textit{Clostridium} and \textit{Enterobacteriacea} counts compared with the control. The
combined administration of *L. paracasei* and FOS stimulated the immune system more than the single administration of lactobacilli. Significant differences between these groups were observed in total counts of CD4⁺ T cells and B cells. The results obtained point out to a synergic effect of *L. paracasei* and FOS on numbers of bacterial populations observed in the feces and the immune system of the weanling pigs.

### 29.2.2 Growth-Promoting Effects

Soon after the introduction of the antibiotics for therapy of bacterial infections in production animals, a growth-promoting effect was observed and antibiotics were used as growth-promoting supplements to the feed of farm animals. The mode of action of antimicrobial growth promoters is still not exactly known, but hypotheses explaining their effect are based on a reduction of the growth of bacteria in the intestinal tract and consecutive protection of nutrients against bacterial destruction, the decrease of the production of toxins by intestinal bacteria, and the reduction in the incidence of subclinical intestinal infections (Butaye et al. 2003). The data that are available for different countries show that the use of antimicrobial agents for growth promotion normally equals or exceeds the usage of antimicrobial agents for therapy of farm animals (Aarestrup 2000). The wide use of antibiotics as growth promoters stimulated the emergence of antibiotic-resistant pathogenic bacteria and contamination of the food chain with residues of antibiotics (Roselli et al. 2005; Vondrusková et al. 2010). Human health can either be affected directly through residues of antibiotics in food of animal origin, or indirectly through the selection of antibiotic resistance determinants that may spread to human pathogens and limit the therapeutic potency of antibiotic.

Until 2006 antibiotic growth promoters were included into feed for piglets during the period from birth to weaning to improve the composition of gastrointestinal microbiota and to prevent the occurrence of PWD (Barton 2000; Sorensen et al. 2009). The use of growth-promoting antibiotics was banned in the European Union (EU) from 2006 in view of reducing antibiotic resistance phenomena in human therapies. Animal production needs alternative means of obtaining similar production benefits to maintain profitability and competitiveness. Ways must also be found to improve the healthiness and safety of animal products reaching the consumer.

Because the use of antibiotics as growth promoters has been banned, research has focused on the development of effective alternatives to maintain of animal health and performance (Castillo et al. 2008). Various natural substances, many of which are commercially available, have been investigated as efficient alternatives to antibiotic growth promoters. At present, probiotics, prebiotics, organic acids, enzymes, phytobiotics, clay adsorbents, and others are under investigation. These materials can exert beneficial effects on the microbiota composition and consequently affect animal health and growth performance (Vondrusková et al. 2010).

Many authors have reported a growth-stimulating effect of probiotic lactobacilli to pigs (Pollmann et al. 1980a). However, some authors did not observe growth improvement with the administration of probiotics. Probiotic microorganisms improve the intestinal microbiota composition and influence digestive processes by the improvement of the microbial population that is beneficial to the host, by enhancing its enzyme activity and improving digestibility and feed utilization. Different *Lactobacillus* species and strains have beneficial effects on feed intake and growth performance of weanling pigs (Mathew et al. 1998; Scheuermann 1993; Lessard and Brisson 1987). The application of *L. acidophilus* (Pollmann et al. 1980b) and *L. reuteri* BSA131 (Chang et al. 2001) tended to improve average daily gain and feed conversion in 4 weeks old pigs. Beneficial effects of probiotic treatment using *B. lactis* HN019 on performance of piglets have been observed (Shu et al. 2001).
In future research, understanding the mechanism by which probiotics improve health and stimulate growth is needed.

29.3 Probiotics for Ruminants

Application of probiotics or direct-fed microbials (DFM) have been employed in ruminant production for over 30 years, and were used primarily in young ruminants to accelerate establishment of the intestinal microbiota to promote gut health. Further advancement led to more sophisticated probiotic feeds that were targeted at improving fiber digestion and reducing ruminal acidosis in mature cattle, aiming improvements in milk yield, growth, and feed efficiency. Probiotics with antibacterial effect against potentially zoonotic pathogens (e.g., \textit{E. coli}, \textit{Salmonella} spp., \textit{Clostridium perfringens}) are an important development. Commonly used probiotics include, but are not limited to, lactobacilli, streptococci, enterococci, bifidobacteria, and propionibacteria (Walker 2007).

In recent years, the use of feed additives containing bacterial and yeast cultures has been increased. Yeast culture was reported to have positive effects on nutrient digestibility and rumen activity. Yeast has been shown to provide nutrients that stimulate the growth of certain rumen microorganisms (probiotics effect) such as the stimulation of rumen bacteria \textit{Selenomonas ruminantium}. \textit{S. cerevisiae} stimulation of rumen bacteria depends on its respiratory activity, which allow it to scavenge O$_2$, thus protecting the strictly anaerobic bacteria. The culture is a yeast-fermented product that contains live and dead yeast cells and the spent culture medium containing the metabolites produced by the yeast during fermentation (Linn and Raeth-Knight 2006). When fed to cattle, yeast cultures have been shown to stimulate cellulolytic bacteria in the rumen, improve fiber digestion, and stabilize rumen pH (Rossi et al. 2006). Yeast also provides vitamins to support the growth of rumen fungi (Hong et al. 2005). Most of these have been shown to be the most active in the lower gut of a ruminant.

Regulatory requirements on application of probiotics in feed of cattle have limited the microbial species that are recognized as safe, such as lactic acid–producing bacteria (e.g., \textit{Lactobacillus}, \textit{Bifidobacteria}, \textit{Enterococcus} spp.), fungi (\textit{Aspergillus oryzae}) or yeast (e.g., \textit{S. cerevisiae}), and Bacillus spores (e.g., \textit{B. subtilis} and \textit{B. lichenformis}). Concerns over the safety of probiotics include deleterious metabolic activation, excessive immune stimulation, and gene transfer of virulence and antimicrobial resistance genes among microorganisms (Agostoni et al. 2004).

29.3.1 Health Benefits and Application

The application of probiotics in cattle is recommended in neonatal calves, during the period postweaning, in time of stress, postpartum, and in disease-induced changes in metabolism (subacute and acute acidosis, methane production in rumen).

Probiotics are extensively used in ruminants to stabilize rumen pH and help in various other important functions that are required for their well-being, milk production and breeding, and produce beneficial enzyme and improve nutrient availability and uptake. The use of probiotics in ruminants is more complicated and often depends on whether the target is to combat acidosis, alter the feed-to-weight conversion, reduce the incidence of disease, or decrease methane production (Krehbiel et al. 2003). The application of probiotics in ruminants is documented in Table 29.2.

Oral administration of lactobacilli generally resulted in an augmentation of innate immune responses (i.e., enhanced phagocytosis and natural killer cell activity), as well as an elevated production of IgA and decreased IgE production in both humans and animals. Bacterial DFM have
been shown to affect the innate, humoral, and cellular arms of the immune system. Modulation of host immunity may represent another mechanism by which DFM promote intestinal health and overall well-being of the host (Erickson and Hubbard 2000; Isolauri et al. 2001). The animal host immune system is capable of mounting both nonspecific (innate) and specific (adaptive) immune response against a variety of pathogens when encountered.

Recently, microorganisms that are not part of the natural microbiota in ruminants have been introduced. This includes Bacillus spores such as *B. subtilis* and *B. licheniformis*, which are not normal inhabitants of gastrointestinal tract and have been suggested to stimulate the immune system as they are perceived as potentially unharmful (Riddell et al. 2010). Species including *B. subtilis* and *B. licheniformis* may be included as one or more of components in probiotic products (Hong et al. 2005).

The general beneficial effects of lactic acid bacteria (LAB) include detoxification of harmful metabolites to improve animal health; displacement of pathogen bacteria from the gut wall through competitive inhibition for attachment, adhesion, or colonization sites in the digestive

<table>
<thead>
<tr>
<th><strong>Microbial Species</strong></th>
<th><strong>Observed Effect</strong></th>
<th><strong>References</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. gallinarum</em> LCB 12</td>
<td>Antibacterial activity</td>
<td>Ohya et al. (2000)</td>
</tr>
<tr>
<td><em>Streptococcus bovis</em> LCB6</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>L. acidophilus</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>L. casei</em></td>
<td>Milk lactation</td>
<td>Gomez-Basauri et al. (2001)</td>
</tr>
<tr>
<td><em>E. faecium</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. cerevisiae</em> CNCM-1077</td>
<td>Microbial colonization</td>
<td>Chaucheyras-Durand and Fonty (2002)</td>
</tr>
<tr>
<td><em>L. acidophilus</em> NPC 747</td>
<td>Antibacterial activity</td>
<td>Brashears et al. (2003a, b)</td>
</tr>
<tr>
<td><em>L. rhamnosus</em> strain GG</td>
<td>Diarrhea</td>
<td>Ewaschuk et al. (2004)</td>
</tr>
<tr>
<td><em>E. faecium</em> EF9296</td>
<td>Antibacterial activity</td>
<td>Marciňáková et al. (2004)</td>
</tr>
<tr>
<td><em>L. casei</em></td>
<td>Diarrhea</td>
<td>Bomba et al. (2006a)</td>
</tr>
<tr>
<td><em>L. acidophilus</em> <em>Propionibacterium freudenreichi</em></td>
<td>Milk lactation</td>
<td>Stein et al. (2006)</td>
</tr>
<tr>
<td><em>Prevotella bryantii</em> 25 A</td>
<td>Early milk lactation</td>
<td>Chiquette et al. (2008)</td>
</tr>
<tr>
<td><em>S. cerevisiae</em></td>
<td>Milk lactation</td>
<td>Robinson and Erasmus (2009)</td>
</tr>
<tr>
<td><em>L. plantarum</em> Chikuso-1</td>
<td>Diarrhea</td>
<td>Kawakami et al. (2010)</td>
</tr>
<tr>
<td><em>Candida</em> sp. CO119</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>B. subtilis, B. licheniformis</em></td>
<td>Enhanced immunity</td>
<td>Riddell et al. (2010)</td>
</tr>
</tbody>
</table>
Lactic Acid Bacteria: Microbiological and Functional Aspects

The tract; stimulation of local immune response in the gut; production of B vitamins; production of lactic acid; and creating a hostile condition for pathogenic bacteria.

The discovery of antibiotics after World War II decreased the interest in probiotics, but they were still used to reestablish the intestinal microbiota following aggressive antibiotics treatments (Chiquette 2009). Probiotics are candidates for antibiotic alternatives (Callaway et al. 2004). Antibiotics destroy undesirable but also beneficial microorganisms and expose the rumen to undesirable metabolic changes. Probiotics are employed in cattle production to reduce the use of antibiotics in neonatal and stressed calves, enhance milk production, prevent ruminal acidosis, improve the feed conversion ratio (FCR), enhance the competitive exclusion towards enteropathogens, and to rapidly establish a stable microbiota in neonatal calves (Krehbiel et al. 2003). The use of antibiotics in animal production may contribute to the emergence of antibiotic-resistant bacteria from cattle industry (Fey et al. 2000), the establishment of new technologies alternative to antimicrobial agents is strongly needed.

29.3.1.1 Use of Probiotics in Neonatal Diarrhea in Calves and Lambs

Inclusion of probiotics in the diet of young calves has been shown to improve performance characteristics, including body weight gain (BWG) and feed conversion as well as average daily gain in the first 2 weeks of life. Primarily in young ruminants, application of probiotics accelerates establishment of the intestinal microbiota involved in feed digestion and promotes gut health.

Gastrointestinal disorders, including diarrhea, are one of the leading causes of mortality and morbidity in neonatal calves, and a reduction in their incidence and duration of diarrhea has been reported in calves consuming probiotics (Kawakami et al. 2010). Neonatal diarrhea is the main cause of calf death and serious economic problem in the cattle industry. *E. coli*, salmonellae, rotaviruses, and coronaviruses as well as cryptosporidia play an important role in the etiology of diarrheal syndrome in calves. In the young, enterotoxigenic *E. coli* appear to be the most frequent diarrhea-causing agent. The first days following birth and the weaning period are two critical periods where calves have been shown to benefit from probiotic addition to their feed. In the neonate, the microbial population of the gastrointestinal tract is in transition and extremely sensitive (Nousuainen et al. 2004).

Probiotics have been used to decrease diarrhea occurrence in many species. Timmerman et al. (2005) conducted an experiment comparing the difference between multispecies probiotics in milk ruminants and found that reduced the incidence of diarrhea in veal calves. Magalhaes et al. (2008) found the addition of yeast to calf starter significantly improved fecal scores, along with decreasing mortality rates in calves experiencing high incidence of diarrhea.

Ewaschuk et al. (2004) used *LGG* isolated from human intestine to maintain viability in the gastrointestinal tract of calves in prevention of diarrhea. This probiotic has been shown to be resistant to acid and bile, have strong adhesive properties to human and rabbit intestinal mucosal cells, suppress bacterial enzyme activity, and produce antimicrobial substances (Lee et al. 2000). Results showed that *LGG* survives intestinal transit in the young calf, produces no D-lactate, and can be administered in an oral rehydration solution. The application of *L. casei* to calves during the first 3 days of age decreased the morbidity and therapy expenses by more than 30% and mortality by more than 50% (Bomba et al. 2006a).

Chaucheyras-Durand and Fonty (2001) reported that the addition of probiotics to the diet of lambs increased the rate at which different bacterial species became established. The role of probiotics under these circumstances is to colonize the gut thereby preventing colonization by enteropathogens that cause diarrhea. Chaucheyras-Durand and Fonty (2002) used probiotic yeast,
S. cerevisiae I-1077, on microbial colonization of the rumen of newborn lambs. This probiotic may be able to accelerate the functionality and/or improve the stability of the rumen ecosystem in young ruminants.

29.3.1.2 Probiotics and Reduction of E. coli O157:H7

Historically, E. coli enterotoxemia has not been considered as a viable target for antimicrobials in cattle because of the complexity of rumen microbiology and the relatively long production cycles in the cattle industry.

Feedlot cattle have been recognized as a host for E. coli O157:H7. This organism appears to be confined to the gastrointestinal tract and is shed in feces. Ohya et al. (2000) developed and studied the effect of a DFM containing lactic acid–producing Streptococcus bovis LCB 6 and Lactobacillus gallinarum LCB 12 isolated from adult cattle on the elimination of E. coli O157:H7 from experimentally infected Holstein calves. Very few studies have looked at E. coli enterotoxemia of food borne pathogens from feedlot cattle (Tkalcic et al. 2003; Zhao et al. 2003). Brashears et al. (2003a, 2003b) demonstrated a significant reduction in the fecal shedding and carcass contamination of feedlot cattle with E. coli O157:H7 using a monostrain probiotic (L. acidophilus, NPC 747).

29.3.1.3 Use of Probiotics in Prevention of Ruminal Acidosis

A probiotic for ruminant animals is a biological preparation that is designed to promote beneficial rumen microbes and to stabilize rumen conditions. The rumen is a complex ecosystem that plays a major role in feed digestion. In adult animals its volume is about 100 liters and it harbors bacteria (10^{11} cells/ml), protozoa (10^5 cells/ml), fungi (10^3 cells/ml), and methanogens (10^9 cells/ml) (Chiquette 2009). The rumen microorganisms play key role in efficient digestion. It is through the rumen that the animal starts deriving energy, protein, and mineral nutrition from pasture and feed. The process is dominated by microorganisms. Ingestion of solid feeds stimulates rumen microbial growth and production of VFA, while calves receiving a liquid diet of milk or milk replacer have minimal development (Heinrichs and Lesmeister 2005).

Ruminal acidosis can be classified clinically as subacute and acute acidosis. In subacute acidosis, elevated levels of short-chain or branched-chain fatty acids, but not lactates, accumulate in the rumen. Acute acidosis consists of high levels of lactic acids in the rumen. Some bacteria with very specific functions in the rumen such as Butyribiofibrosolvens, which produce conjugated linoleic acids from linoleic acid, have been proposed as probiotics for ruminants (Fukuda et al. 2006).

Lactobacilli may be useful in reducing ruminal acidosis. A strain of L. acidophilus has been shown to reduce total d/l lactate levels and sustain a ruminal pH of 6.0 (Krehbiel et al. 2003). The microbial changes associated with low ruminal pH are an increase in the number of pH-tolerant bacteria (S. bovis—lactate producer and Megasphaera elsdenii—lactate user). When ruminal pH is below 6.0, the activity of cellulolytic bacteria is seriously decreased and the number of protozoa declines. Ruminal acidosis can be defined as a low ruminal pH of below 5.6 and high ruminal VFA concentrations (Collins et al. 2009). LAB, including members of genera Lactobacillus and Enterococcus are the most extensively studied as probiotics on the pH-stabilizing effect in the rumen (Nocek and Kautz 2006). Production of bacteriocins by some probiotic bacteria (such as E. faecium) allows them to control the growth of certain pathogens in the rumen. Peterson et al. (2007) reported that L. acidophilus strains reduced the shedding of E. coli O157:H7 in cattle.

Fungi (A. oryzae) and yeasts (S. cerevisiae) are used as DFM in cattle. It seems to be effective in preventing ruminal acidosis in cattle. Application of live yeasts in feed to cattle can affect on
the host by removing oxygen in the rumen and boosting the anaerobic environment for anaerobes and subsequently increases the number of lactate-utilizing bacteria that are believed to control the acidosis (Collins et al. 2009).

29.3.1.4 Use of Probiotics in Control of Methane Production

Methanogens in the rumen convert carbon dioxide into methane by reduction with hydrogen. Methanogens play an important role in the rumen by actively scavenging hydrogen, which is detrimental to rumen digestion (Takahashi et al. 2005).

Different nutritional components and specific rumen microorganisms influence methane production in the rumen. Acetate, carbon dioxide, and hydrogen are the major methane precursors in the rumen, and these metabolites are produced mainly by the breakdown of carbohydrates, in particular cellulose. Therefore, rumen metabolism and subsequent methane production is expressed as the sum of all the different metabolisms depending on the level of carbohydrates provided (Collins et al. 2009). The complete detailed biochemistry of methane production in the rumen has been presented by Mitsumori (2008). In vitro data using twin strains of S. cerevisiae demonstrated a small reduction in methane production after 24 h incubation of the yeast in mixed rumen fermentation vessel (Lila et al. 2004).

29.3.1.5 Probiotics as Additive to Silage Feed for Ruminants

The use of silage for animal feeding has sometimes been associated with pathological problems, including listeriosis. Marciňáková et al. (2004) studied the probiotic properties of E. faecium EF 9296 strain isolated from silage as potential silage probiotic to control, especially listerial contamination in silages. Consequently, a new probiotic strain, E. faecium EF 9296, was used in grass an ensiling as inoculant (Marciňáková et al. 2008). The silage inoculation resulted in a more rapid drop of pH, higher level of lactic acid, and in decrease of the acetic and butyric acid concentration. This strain represents a promising silage additive to produce of silage good quality and/or to prevent silage contamination.

29.3.2 Probiotics and Production of Milk and Beef

Research on the effect of bacterial probiotics on milk yield and composition has been very limited. Chiquette et al. (2008) reported increased production of fermentation and milk fat percentage when a newly isolated bacterial strain (Prevotella bryantii 25A) was fed to dairy cows from 3 weeks prepartum to 7 weeks postpartum. Gomez-Basauri et al. (2001) observed an increase in milk production (0.73 kg/day) when feeding cows a mixture of L. acidophilus, L. casei, and E. faecium. Other experiments have been conducted with combinations of fungal cultures and LAB (Block et al. 2000). Milk yields were increased by 1.08 and 0.90 kg/day, respectively, when lactating cows were fed S. cerevisiae in combination with L. acidophilus or 5 × 10⁹ colony-forming units (CFU) of yeast in combination with 5 × 10⁹ CFU of L. plantarum/E. faecium. Stein et al. (2006) reported an 8.5% increase in 4% fat corrected milk in cows receiving 6 × 10⁹ Propionibacterium/day from 2 weeks prepartum to 30 weeks postpartum. Raeth-Knight et al. (2007) failed to observe any effect on milk yield or composition or dry matter intake when feeding dairy cows (averaging 74 ± 32 days in milk) a combination of L. acidophilus (1 × 10⁹ cells/day) and P. freudenreichi (2 × 10⁹ cells/day).

The use of probiotics in beef production can be separated into two categories: a) protection of stressed calves and b) continuous dosing of feedlot cattle. Neonate calves are often stressed, and
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Research has shown that stress can alter the gut microbiota population. Stressed calves that experience diarrhea have a lower population of lactobacilli in their intestinal tract and such stressors include weaning, transport, vaccination, castration dehorning, and new environment. Interest in the effects of feeding probiotics on animal health and performance has increased due to concern regarding the use of antibiotics and other growth stimulants in the animal feed industry.

29.4 Probiotics for Poultry

The poultry industry has during the past two decades been one of the most dynamic and expanding animal husbandry sectors in the world. Raising of healthy animals is very demanding, due to the fact that this process is affected by many factors. In large-scale rearing facilities, where poultry are exposed to stressful conditions, problems related to enteric diseases result in lost productivity, increased mortality in flocks, and also potential contamination of poultry products, which leads to human food safety concerns. Prevention and control of diseases have during recent decades led to a substantial increase in the use of veterinary medicines. However, the utility of antimicrobial substances in poultry may have a negative impact on public health through the increase of resistant bacteria or bacteria producing resistant genes that enter the human organism directly or indirectly. One of the most fundamental changes in the EU legislation is a ban on the use of antimicrobial feed growth promoters in all EU states from January 2006 (Regulation EC 1831/2003 of European Parliament and Council from September 2003 about additives used by farm animals). Today, in developed countries, emphasis is put primarily on safety and healthiness of poultry products reaching the consumer and also on minimizing of any negative impact of the poultry industry on the environment. To achieve these objectives along with maintaining of high productivity of farms, preparations of biotechnological origin, such as probiotics, are used for the replacement of antibiotic growth promoters.

29.4.1 Health Benefits and Application

The probiotic species belonging to the genera *Lactobacillus*, *Streptococcus*, *Bacillus*, *Bifidobacterium*, *Enterococcus*, *Aspergillus*, *Candida*, and *Saccharomyces* have been studied for their impact on growth performance, modulation of intestinal microbiota and pathogen inhibition, immune modulation, meat quality, and egg production of poultry. However, it is difficult to directly assess different studies using probiotics because the efficacy of a probiotic application depends on many factors (Patterson and Burkholder 2003), such as species composition and viability, administration level, application method (e.g., spraying, feed, or water), frequency of application (e.g., once, intermittent, or continuous), overall diet, bird age, overall farm hygiene, and environmental stress factors (e.g., temperature, stocking density).

29.4.1.1 Effect on Modulation of Intestinal Microbiota and Pathogen Inhibition

The autochthonous microbiota is considered to act as one of the body’s natural defenses, and consists of the population of mostly nonpathogenic bacteria normally residing in the GIT. This population is considered to play an important role in the development of “colonization resistance” against potential pathogens. In the last five decades, increased knowledge of the factors that influence the activities of microorganisms in the alimentary tract has helped define the critical role of
symbiotic organisms. Probiotics, competitive exclusion, and DFM feed supplements can be used as strategic tools for managing these microbial populations and inhibition of pathogens.

Profiling of the cecal microbiota using temporal temperature gradient gel electrophoresis in combination with principal component analysis demonstrated beneficial impact of the probiotic treatment on the overall bacterial community as well as on the Lactobacillus population (Gérard et al. 2008). Supplementing the diet of chicks and ducks with one dose of L. fermentum increased the DNA copies of Lactobacillus spp. and Firmicutes in the feces, whereas the population of Bacteroidetes remained stable or slightly decreased. As a result, the Firmicutes/Bacteroidetes ratio increased after Lactobacillus spp. inoculation (Angelakis and Raoult 2010). Strompfova et al. (2005) determined the influence of orally administered L. fermentum AD1 strain on intestinal microbiota, of conventional Japanese quail. The results demonstrated that the 4-day application of this strain significantly increased the population of LAB in feces and cecum of quail and significantly decreased the counts of E. coli in feces. Also, Watkins and Kratzer (1983) and Francis et al. (1978) reported that chicks and turkeys dosed with Lactobacillus strains had lower numbers of coliforms in the cecum and small intestine than the control. Kralik et al. (2004) reported a decrease in the number of bacteria of the Enterobacteriaceae family and coliforms, to about 90% of the control sample after 42 days of supplementing water with a probiotic containing 5 × 10^9 CFU/g of E. faecium M-74. However, they did not find any statistically significant differences in relation to bacteria of Staphylococcus sp., Bacillus sp., and Clostridium sp. The probiotic product containing L. reuteri, E. faecium, B. animalis, P. acidilactici, and L. salivarius resulted in a beneficial modulation of the cecal microbiota, as evidenced by the significant (p ≤ .05) increases in the concentrations of bacteria belonging to Bifidobacterium spp., Lactobacillus spp., and gram-positive cocci and significantly higher specific activities of α-galactosidase and β-galactosidase compared with the control and antibiotic treatment (Mountzouris et al. 2007). Lan et al. (2004) also found that 10^6 CFU L. agilis and L. salivarius enriched the diversity of Lactobacillus biota in the chicken jejunum and cecum by increasing the abundance and prevalence of Lactobacillus spp. inhabiting the intestine. The same probiotic treatment, when used for 40 days in chickens, reduced the number of Enterobacteriaceae, whereas the number of lactobacilli and enterococci remained stable (Lan et al. 2003). Other studies using multistrain–single species (Jin et al. 1998) as well as multistrain–multispecies (Priyankarage et al. 2003) probiotics have shown no significant changes in the gut microbiota profile of broilers.

Wu et al. (2009) reported that the diet containing a dried B. subtilis culture (DBSC) at 250 mg/kg significantly improved the cecal ecosystem of goslings by increasing the probiotics population and VFA concentration. Lactobacillus and Bifidobacterium counts were significantly increased and the harmful bacteria (p < .05) was markedly reduced. The total VFA, acetic, and butyric acid concentration increased on DBSC supplementation resulting in lower pH. The application S. cerevisiae at 0.4% or 0.8% into laying hen diets increased lactobacilli counts and reduced bacterial levels of E. coli, Klebsiella sp., Staphylococcus sp., Micrococcus sp., Campylobacter sp., and C. perfringens (Hassanein and Soliman 2010).

The majority of poultry competitive exclusion experiments have focused on the two main zoonoses in which poultry are a major reservoir: Salmonella spp. and Campylobacter spp. However, recent work has focused on emerging or opportunistic pathogens found in poultry with, for example, C. perfringens, the causative agent of necrotic enteritis in poultry, and E. coli O78:K80, the cause of avian colibacillosis. Since Nurmi and Rantala (1973) first applied the concept of competitive exclusion in poultry to protect chickens against Salmonella infection by inoculating them with microbiota from adult birds, numerous studies have demonstrated that the competitive exclusion effects of probiotics can protect hosts against pathogens such as Salmonella...
Typhimurium, Salmonella Gallinarum, C. jejuni, C. perfringens, E. coli O157:H7, and E. coli O78:K80 (Schoeni and Wong 1994; Nisbet et al. 1998; Corrier et al. 1998; Stavric et al. 1993; Timmerman et al. 2004; Casey et al. 2007; Zhang et al. 2007). Many of the competitive exclusion treatments are either defined or undefined cecal contents or multiple bacterial species derived from cecal contents. Some research has focused on early prevention of colonization, which can help limit pathogen populations; other studies have demonstrated long-term colonization benefits of DFM products. Single-strain probiotics, L. reuteri, L. salivarius CTC2197, L. johnsonii FI9785, L. acidophilus, E. faecium, B. subtilis, and B. longum PCB 133 were shown to decrease the colonization of chicks and turkeys by Salmonella, E. coli, C. jejuni, and C. perfringens (Edens et al. 1997; Morishita et al. 1997; Pascual et al. 1999; La Ragione et al. 2001, 2004; La Ragione and Woodward 2003; Santini et al. 2010). Vicente et al. (2008) and Higgins et al. (2008) both reported the use of commercial Lactobacillus preparation FM-B11 to inhibit Salmonella Enteritidis in commercial broilers. Willis and Reid (2008) showed that C. jejuni was present at a lower level in broiler chickens fed with a standard diet supplemented with a probiotic formulation containing L. acidophilus, L. casei, B. thermophilus, and E. faecium (10^8 CFU/g) with respect to the control. Different Lactobacillus strains have demonstrated to have a protective effect on raw chicken meat against L. monocytogenes and Salmonella Enteritidis (Maragkoudakis et al. 2009).

29.4.1.2 Effects on Immune Response

Probiotic bacteria can exert immune-modulating activities through their interactions with the host immune system. These interactions may lead to enhancement of innate immunity and antigen-specific antibodies, activation or suppression of T cells, and changes in cytokine expression profiles. Moreover, probiotics are able to induce the expression of antimicrobial peptides by host cells.

The immune-modulating properties associated with probiotic application in poultry are well known. Haghighi et al. (2006) showed significant increases in natural serum IgA, IgG, and IgM antibodies specific for tetanus toxoid after a single intragastric application of probiotic containing L. acidophilus, B. bifidum, and E. faecalis in immunized chickens. Likewise, Koenen et al. (2004) reported increases in total IgG and IgM titers in chickens receiving L. plantarum, L. paracasei, and also observed increased phagocytic activity of gut-associated immunity cells toward Salmonella. On the other hand, Midilli et al. (2008) showed the ineffectiveness of additive supplementation of probiotics on serum IgG concentration of broilers. Another study by Yurong et al. (2005) reported increases in the number of Ig-producing cells (IgM, IgG) detected in Peyer’s patches and the cecal tonsils of chicks by days 7 and 10, respectively, following administration of a probiotic culture in the drinking water containing B. subtilis, Candida utilis, and L. acidophilus. The density of microvilli and length of the cecal tonsils was also increased following 3 days of probiotic administration. Significant increases in heterophil degranulation and oxidative burst were observed with B. subtilis–, Lc. lactis–, and L. acidophilus–treated chicks (Farnell et al. 2006). In terms of immunological response, broilers provided feed supplemented with B. subtilis PB6 had significantly heavier bursas, heterophils with higher in vitro phagocytosis for E. coli, and lower ileal E. coli populations, indicating a potentiating role of B. subtilis PB6 as a probiotic on the chicken innate immune system (Teo and Tan 2007).

In splenocytes and cecal tonsil cells STAT2 and STAT4 genes were highly induced, and the expression of STAT2, STAT4, IL-18, MyD88, IFN-α, and IFN-γ genes were up-regulated in cecal tonsil cells after treatment with L. acidophilus DNA (Brisbin et al. 2008). Haghighi et al. (2008) investigated the underlying immunological mechanisms of the action of probiotics against
colonization of the chicken intestine by Salmonella Typhimurium. There was no significant difference in IL-6 and IL-10 gene expression in cecal tonsils of chickens belonging to various treatment groups. Salmonella Typhimurium infection resulted in a significant increase in IL-12 expression in cecal tonsils on days 1 and 5 postinfection. However, when chickens were treated with probiotics before experimental infection with Salmonella, the level of IL-12 expression was similar to that observed in uninfected control chickens. Treatment of birds with probiotics resulted in a significant decrease in IFN-γ gene expression in cecal tonsils of chickens infected with Salmonella compared with the Salmonella-infected birds not treated with probiotics. By contrast, Fujiwara et al. (2009) observed no statistically significant differences in the expression of IFN-γ, IL-3, and IL-4 when birds were fed diets with or without B. subtilis–fermented soybean. Dalloul et al. (2005) suggested a positive impact of the probiotic in stimulating some of the early immune responses against Eimeria acervulina, as characterized by early IFN-γ and IL-2 secretions, resulting in improved local immune defenses against coccidiosis. Chichlowski et al. (2007) investigated intestinal inflammation status through mucosal cytokine production on broiler chicks fed the DFM PrimaLac. The reverse transcription-PCR data demonstrated that DFM consortium numerically altered expression of IL-6 (decreased), a pro-inflammatory cytokine, and IL-10 (increased) an anti-inflammatory cytokine within the ileum of 19-day posthatch broilers. On the other hand, another pro-inflammatory cytokine, IL-1β, was not affected by the DFM diet.

L. reuteri, L. salivarius, L. acidophilus, and cecal microbiota isolated from three 35-week-old breeders and supplied by oral gavage to chicks, challenged or not with Salmonella Enteritidis, demonstrated through immunohistochemistry the capacity to stimulate the immune system in the form of leukocytic infiltrate by the CD3+, CD4+, and CD8+ lymphocytes in the intestinal epithelium and in the intestinal lamina propria of chicks (Noujaim et al. 2008). Similar results were obtained by Vervelde et al. (1998) who identified, also by immunohistochemistry, a great quantity of leukocytic infiltrate by CD3+ lymphocytes constitutes, principally, of CD4+ and CD8+ cells in the epithelium and in the lamina propria of chick intestine, 7 days after the treatment realized with a mixture of recombinant antigen of Eimeria and choleric toxin. Likewise Choi et al. (1999) described the alterations of subpopulations of T cells, among them the CD4+, CD8+, TCR1, and TCR2 lymphocytes, as well as the transcription of IFN-γ and TGF-β4 mRNA in the intestine of chicks, after oral inoculation of E. acervulina. According to Songserm et al. (2002), in addition to bacterial stimulus, the increase in chick age is responsible for the influx of lymphocytes into the intestinal epithelium of chicks.

Akbari et al. (2008) demonstrated that infection of young chicks with Salmonella Typhimurium significantly increases the expression of several of the antimicrobial peptide genes in cecal tonsils. Furthermore, when chickens were treated with probiotics before Salmonella infection, the expression of avian β-defensin and cathelicidin genes were reduced to levels comparable with those seen in the negative control group.

29.4.2 Effects on Growth Performance

It is important to consider the economic implications of probiotic application. Increasing animal BWG and improving FCR are measures that can indicate increased profitability for the producer. The inclusion of probiotics may positively affect these measures in poultry. It was speculated that the beneficial impact of probiotic supplementation on poultry performance would be the outcome of a fine tuning of the complex gut ecosystem, resulting in improved digestive function, intestinal environment, and broiler health. Reports on the efficacy of probiotic products have been variable with positive effects on poultry performance reported by some and no or neutral
effects reported by others. The research focused on the use of multispecies probiotics and various strains of *Lactobacillus*, *B. faecium*, and *Bacillus*. Timmerman et al. (2004, 2005) provided evidence that multispecies probiotics are more effective than monospecies probiotics and also that species-specific probiotics elicit different health effects than do probiotics derived from another host species. The combination of six *Lactobacillus* strains that were isolated from fresh digesta and intestinal tissue samples of healthy chickens had improved the survival rates of broilers in controlled trials by the addition of probiotics to the drinking water. Efficacy of the *Lactobacillus* strains was reduced under high-production conditions (Timmerman et al. 2006). Jin et al. (1998) noted a dose-dependent response with *Lactobacillus* application. The authors described reduced mortality in chickens receiving 0.10% (wt/wt) *Lactobacillus* culture (12 strains of *Lactobacillus* isolated from chicken intestine, which belong to four species—*L. acidophilus*, *L. fermentum*, *L. crispatus*, and *L. brevis*) from 8.2% in control birds to 3.2% in treated animals. These animals also demonstrated superior FCR. However, higher doses (0.15% wt/wt) resulted in lower productivity, near that of the control group receiving no *Lactobacillus* culture. Chickens receiving the low 0.05% (wt/wt) DFM dose demonstrated performance values between the 0.10% and 0.15% dosed birds. Mountzouris et al. (2007) investigated the efficacy of the multibacterial species probiotic product Biomin Poultry5Star (containing *Lactobacillus*, *Bifidobacterium*, *Enterococcus*, and *Pediococcus* strains) in broilers nutrition. Probiotic treatment in feed and water (PFW) displayed a growth-promoting effect that was comparable to avilamycin treatment. FCR in avilamycin treatment was significantly better (*p ≤ .01*) than the control treatment, whereas treatments PFW and probiotic in feed (PF) were intermediate and not different from antibiotic. However, the higher broiler body weight obtained in treatment PFW compared with treatment PF might be the result of the overall higher probiotic intake in the former due to the additional water application. Each bird in treatment PFW had an additional daily intake of 10⁸ CFU probiotic bacteria during application via drinking water. However, contrary to their previous work, Mountzouris et al. (2010) reported that the best growth performance in broilers was achieved with a 10-fold lower probiotic dietary inclusion and actual intake level compared with previous work. A possible explanation for the difference between the two studies could be the fact that in the second study, a coccidiostat-free diet was used. A coccidiostat such as salinomycin sodium, used in previous work, is an ionophore that has antibiotic properties and as a result a higher probiotic concentration might have been necessary to achieve a growth-promoting effect. Primalac, a DFM product that contains *L. acidophilus*, *L. casei*, *B. thermophilum*, and *E. faecium*, was evaluated in various experiments (Nayebpor et al. 2007; Grimes et al. 2008; Russell and Grimes 2009), resulting in improved turkey and broiler live performance. Kabir et al. (2004) reported the occurrence of significantly (*p < .01*) higher live weight gains and carcass yield (weight of leg and breast) in broiler chicks fed with the multistrain probiotic Protexin® Boost (containing *L. plantarum*, *L. bulgaricus*, *L. acidophilus L. rhamnosus*, *B. bifidum*, *S. thermophilus*, *E. faecium*, *Aspergillus oryzae*, and *Candida pintolopessi*) on the 2nd, 4th, and 6th week of age both in vaccinated and unvaccinated birds. Supplementation of broiler starter and finisher diets with Protexin 100 g/t in starter and 50 g/t in finisher rations was beneficial in terms of weight gain, feed efficiency, and economic viability.

On the other hand, Alkhalf et al. (2010) investigated the effects of a monospecies probiotic Bactocell® (*P. acidilactici*) on broiler growth performance. Probiotic supplementation significantly increased the body weight and daily weight gain of broiler chicks at late ages (3–6 weeks). Also, the birds fed on probiotic levels 1 and 0.8 g/kg diet exhibited higher body weight among chicken groups at 6 weeks of age. At 3, 4, 5, and 6 weeks of age, there were significant differences in the daily feed consumption and in the means of feed conversion among the probiotic treatment groups and also between the probiotic groups and the control group. The probiotic group receiving
1.6 g/kg ration showed less FCR than the other levels of probiotic groups as well as control group. On the other hand, the control group showed higher FCR than the probiotic treatment groups at all times. Edens et al. (1997) showed that *in vivo* and *ex vivo* administration of *L. reuteri* resulted in an increased villus height, indicating that probiotics are potentially able to enhance nutrient absorption and thereby improve growth performance and feed efficiency. A single dose of *L. fermentum* or *Lactobacillus* spp. (Autruche 4) administered intragastrically improves WG and FCE of broiler chicks (Khan et al. 2007). These results were confirmed by Angelakis and Raoult (2010) who inoculated one dose of *L. fermentum* (originally isolated from an ostrich) in newborn chicks and ducks. The animals inoculated with *Lactobacillus* displayed a significant increase not only in their body weight but also in their liver mass. The supplementation of 10^6 CFU/g of *L. reuteri* Pg4 in the feed of broiler chicks from 0 to 21 days of age increased body weight and ileal villus height (Liu et al. 2007).

The studies in agriculture showed that probiotics demonstrated greater potential in lower-performing animal-rearing facilities than in those with near-optimal animal performance. Torres-Rodriguez et al. (2007) evaluated the animal performance of turkeys raised under a variety of conditions. The results provided by this trial suggest that administration of the commercial *Lactobacillus*-based probiotic (FM-B11) to turkeys raised under suboptimal conditions increased the average daily gain and market body weight (BW), representing an economic alternative to improve turkey production. The observed effects seemed to be due to better response in subpopulations of flocks with a fair to poor performance history, whereas those with a history of good performance seemed to respond less favorably to the probiotic supplementation. Zulkifi et al. (2000) found that diets containing *Lactobacillus* cultures not only provided enhanced BWG but also improved FCR to chickens reared under stressful environments. On the contrary, probiotics do not always result in enhanced bird productivity when applied in poultry-rearing operations. O’Dea et al. (2006) tested the effect of two commercially available probiotics (containing *L. acidophilus*, *E. faecalis*, and bifidobacteria) on the production efficiency of broiler chickens hatched from the same breeder flock at three different ages (28, 43, and 57 weeks). The objectives of this experiment were to determine if the effectiveness of these products in broilers varied with breeder flock age. There were no significant differences in broiler BW or feed conversion between the probiotic treatments (in drinking water, spray at hatch, in feed) and the control group in any of the trials. Probiotics had no effect on chick quality or production efficiency in broilers produced by the breeder flock ages examined. The lack of positive effect on BW or FCR in chickens fed DFM has also been reported elsewhere (Watkins and Kratzer 1984; Maiolino et al. 1992).

Many factors make *Bacillus* a good candidate for probiotic use; it produces organic acids, possesses the capacity to sporulate, secretes enzymes, and is easily cultured in bulk. In addition, in the spore form, it is more resistant to extreme temperatures, which enables inclusion in the pelleting process used in production of chicken feeds. The administration of *B. coagulans* ZjU0616 via the basal diet had beneficial effects on final weight, daily weight gain, FCR, and survival rate of Guangxi Yellow chicken (Zhou et al. 2010). Wu et al. (2008) reported that the diet containing a DBSC at 250 mg/kg offered large benefits to the growth performance of goslings, and these benefits could equal or exceed those from flavomycin. Also, Jin et al. (1996) reported that diets supplemented with 0.1% *B. subtilis* powder could increase weight gain in 10-day-old male Arbor Acres broiler chicks in a high-temperature and high-humidity environment. Mutuş et al. (2006) investigated the effect of diets supplemented with *B. licheniformis* and *B. subtilis* (BioPlus 2B) on morphometric parameters and yield stress of the tibia. They found that thickness of the medial and lateral wall of the tibia, tibiotarsal index, percentage ash, and P content were significantly improved by the probiotic.
Results of studies with *S. cerevisiae* fed to chickens have not been consistent. Many authors (Ignacio 1995; Hooge et al. 2003; Stanley et al. 2004) reported that feeding yeast to chicks improves BW gain and feed-to-gain ratio. Other authors reported that active dry yeast effectively increases BW gains without affecting the feed-to-gain ratio in broiler chicks or by contrast, supplementation of yeast to broiler diets improves feed-to-gain ratio but not growth rates (Onifade et al. 1999; Kumprechtova et al. 2000; Karaoglu and Durdag 2005). Zhang et al. (2005) evaluated the effects of *S. cerevisiae* cell components (whole yeast, *S. cerevisiae* extract, *S. cerevisiae* cell wall) on the growth performance in Ross broilers. During the 0–3-week period, a lower feed-to-gain ratio (*p* ≤ .05) was observed in *S. cerevisiae* cell wall–fed birds compared with the control, whereas whole yeast and *S. cerevisiae* extract–fed birds had intermediate values. BW gain was not significantly different across treatments. From 4 to 5 weeks of age, the BW gains by whole yeast and *S. cerevisiae* cell wall–fed birds were greater (*p* ≤ .05) than those of the control birds, and BW gain in *S. cerevisiae* extract–fed birds was intermediate. Chen et al. (2009) observed the beneficial effects of combined two-stage fermentation feed inoculated with high proteolytic capacity *B. subtilis* var. *natto* N21 in the first stage, and high acidic capacity Y10 in the second-stage fermentation on BW, weight gain, feed intake and gross energy availability (*p* < .05) in 21- and 39-day-old chickens. Similar improvements in the growth performance had been reported for poultry receiving 0.5% *B. subtilis* fermentation product (Santoso et al. 2001).

### 29.4.3 Effects on Meat Quality and Egg Production

The demand of safe and qualitative meat and egg production on the poultry market has considerably increased nowadays. The producers are eager to use natural and safe nonchemical forage supplements, which positively affect animal health, increase their productivity, and improve quality of the production.

The application of probiotic Protexin in broiler chicks with age ranging from 1 to 35 days showed significant differences in chemical composition, including moisture percentage, CP, crude fat, and crude ash between the probiotic-treated groups and the control (Mahmood et al. 2005). A moderate impact of *E. faecium* M-74 was observed in the carcass yield (80.1% vs. 79.1%), a percentage of carcass trunk in live weight (74.2% vs. 73.6%), and a proportion of breast muscle on bone in live weight in particular (26.5% vs. 24.6%) at 84 days of age in favor of the experimental turkeys compared with the control ones (Chmelnična et al. 2003). Supplementation of probiotics improved the sensory characteristics and microbiological quality of dressed broiler meat at prefreezing and postfreezing storage (Kabir et al. 2005). Zhou et al. (2010) evaluated the effect of *B. coagulans* ZJU0616 with different concentrations on meat quality of Guangxi Yellow chicken. The results showed no significant difference in chemical composition of meat. However, the probiotic had beneficial effects on shear force in raw breast meats of male broilers. The lowest percentage of drip loss was found in the group supplemented with the probiotic at 2.0 × 10⁶ CFU/g. It coincided with the results in shear force and showed positive effects of *B. coagulans* ZJU0616 on meat quality. There are trials showing that enrichment of diets with yeast could favorably improve the quality of meat from broilers. For example, edible meats from broiler chicks fed a diet containing chromium-enriched *S. cerevisiae* or *S. cerevisiae* cell wall and extract exhibited increased tenderness (Zhang et al. 2005) and increased water-holding capacity (Lee et al. 2002). The *S. cerevisiae* cell wall, which contains α-glucan, carboxymethylglucan, mannans, and some proteinaceous substances, has been reported to display relatively good antioxidative properties (Tsiapali et al. 2001). On the other hand, Loddi et al. (2000) reported that neither probiotic nor antibiotic affected sensory characteristics (intensity of aroma, strange aroma, flavor, strange flavor, tenderness, juiciness, acceptability, characteristic color, and overall aspects) of breast and leg meats.
Nahashon et al. (1996) reported positive correlations between *Lactobacillus* diets and nitrogen and calcium retention, and egg mass, and between fat, nitrogen, calcium, and phosphorus retention and body weight gain, and calcium and phosphorus retention, and egg mass, respectively. Dietary supplementation of *B. coagulans* at 100 mg (6 × 10^8 spore) kg⁻¹ diet significantly increased egg shell weight, shell thickness, and serum calcium of White Leghorn layer breeders (Panda et al. 2008). Slight improvement in eggshell thickness in hens supplemented with probiotics for 10 weeks during the peak period was reported by Nahashon et al. (1994) and Mohan et al. (1995). The authors stated that this beneficial effect may be attributable to a favorable environment in the intestinal tract, which may have helped to absorb more calcium. It is known that probiotics might improve the content of calcium, phosphorus, carotenoid, and albumen in serum of layers. The acidic environment produced by the active probiotics facilitates the ionization of minerals, which is essential for their absorption (Ashmead et al. 1985). Haddadin et al. (1996) reported that cholesterol levels in yolks were decreased by 18.8% when laying hens were fed with up to 4 × 10^6 CFU *Lactobacillus* per gram of feed for a 48-week period. Dietary supplementation of a commercial probiotic (BioPlus 2B) and a DBSC increased egg production, but decreased the damaged egg ratio, egg yolk cholesterol, and serum cholesterol and triglyceride levels (Kurtoglu et al. 2004; Xu et al. 2006). It has been reported that probiotics reduce the plasma cholesterol and triglyceride, confirming the important roles of gastrointestinal microorganisms in recycling of lipids. Probiotic supplementation was not able to completely alleviate a marginal amino acid deficiency in laying hens but improved feed intake-to-egg mass ratios during 8 weeks of the 12-week study (Applegate et al. 2009). Inclusions of yeast into laying hen diets improved egg production and egg weight, enhanced egg shell strength, and reduced soft or broken eggs (Park et al. 2001, 2002).

### 29.5 Probiotics for Other Farm Animal Species

Probiotics can be effectively used also in other farm animals such as honeybees, fur-bearing animals, rabbits, and horses.

#### 29.5.1 Honeybees (*Apis mellifera*)

First attempts have been made with the use of probiotics in honeybees, but in comparison to other animals the knowledge regarding their use in invertebrates is poor.

Honeybees are economically very important especially due to pollination, but like other animals, they can acquire different diseases. American foulbrood (AFB) belongs to the most serious contagious bacterial diseases that affect many beekeepings worldwide. In the EU and some other countries, on the basis of veterinary legislation, the positive colonies are eradicated with high financial loses and the use of antibiotics for the treatment and prevention of AFB is banned. However, in the United States, Canada, most of the countries in South America and East Asia, antibiotics are used both for the therapy and prevention of AFB, and they leave residues in bee products. Moreover, antibiotics are effective only against vegetative forms of *Paenibacillus larvae*, the causative agent of AFB, but spores remain ongoing reservoir of infection (Rada et al. 2009). One of the novel possibilities to fight this disease without drug administration is the use of probiotics for its prevention.

Since the health status of honeybees is strongly dependent on the microorganisms inhabiting the colony, probiotics can positively influence composition of their microbiota. In addition to *Bacillus* sp., lactobacilli and bifidobacteria are the most common bacterial species isolated from the
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LAB are well-known beneficial bacteria used for probiotic purposes, which produce organic acids and other antimicrobial substances inhibiting a wide range of pathogens (Ouwehand et al. 2002). In honeybees, LAB also play an important role in the production of bee bread, which is fermented from the bee pollen by LAB contained in nectar regurgitated from the honey stomach (Vásquez and Olofsson 2009). Furthermore, strong inhibitory activity of LAB against *P. larvae* was confirmed by several authors (Toporčák et al. 2008; Canganella and Balsamo 2008; Forsgren et al. 2010). For these reasons LAB, particularly *Lactobacillus* sp., are the most frequently used bacteria for bee probiotics. On the basis of well-known selection criteria used for development of novel probiotics, potential probiotic LAB are mostly isolated from intestine and honey stomach of bees, pollen, honey, or hive environment; then they are tested for their inhibitory activity against bee pathogens, technological properties, survival in hive conditions; subsequently characterized; and eventually tested for further required properties (e.g., survival in different application forms such as royal jelly, pollen, sugar, or honey solutions).

Toporčák et al. (2008) isolated from digestive tracts and honey stomachs of healthy adult honeybees *L. brevis* and *L. plantarum*, which significantly inhibited *P. larvae*. Both lactobacilli showed good technological properties—survival of long-term freezing storage and good growth properties with pH decrease after 24 h to pH 4 for *L. plantarum* and pH 4.7 for *L. brevis*. It has been shown that germination of bacterial spores is inhibited at around pH 4.2, which can be formed by organic acids produced by LAB (De Vuyst and Vandamme 1994). Production of organic acids from homofermentative *L. plantarum* reached a high concentration of lactic acid (269.2 ± 14.30 mmol/l) and heterofermentative *L. brevis* reached the highest concentrations of acetic acid (73.9 ± 5.87 mmol/l), lactic acid (42.9 ± 3.29 mmol/l), and acetacetic acid (27.2 ± 1.6 mmol/l). The possibility to administer these probiotic lactobacilli through the honey or sucrose solutions into the apiary was tested by monitoring their survival in 12.5%, 25%, and 50% solutions. Both strains survived 3 days in 12.5% and 25% solutions of honey in high numbers (approx. 10⁷ CFU/ml), but after 7 days of incubation no viable bacteria were found in any of the tested concentrations of sucrose and honey solutions.

Forsgren et al. (2010) isolated 11 LAB phylotypes (members of genera *Lactobacillus* and *Bifidobacterium*) from honey stomachs of honeybees and tested their inhibition activity against *P. larvae*. The combination of all isolated LAB resulted in total inhibition of all tested *P. larvae* strains, and therefore this combination was also used in the infection bioassays. Since young larvae are infected by contaminated food and the bacterial spores germinate and bacteria multiply in the midgut, probiotic LAB were administered to the larval food composed of 50% royal jelly and 50% of a solution consisting of D-glucose (12%) and D-fructose (12%). The addition of LAB mixture to the larval food significantly reduced the number of infected larvae (p = .0007) and their mortality. Gontarski (1960) observed that live LAB are present in high numbers in fresh bee bread, which can be a natural source of these bacteria for larvae. If the composition of healthy microbiota is affected, for example, by the application of antibiotics, lack of food, or decreased immunity, pathogenic microorganisms can predominate and cause disease.

Not only LAB have a positive impact on honeybee health and have the potential to be probiotic. Evans and Lopez (2004) found out that some honeybee symbiotic bacteria, most of them belonging to genera *Bacillus* sp., can stimulate immune response of honeybees and thus increase resistance to pathogens. Evans and Armstrong (2006) have subsequently confirmed the antagonistic effect of such symbionts (in addition to *Bacillus* sp., including members of genera *Stenotrophomonas*, *Brevibacillus*, and *Acinetobacter*) against *P. larvae*. Reynaldi et al. (2004) isolated strains of *Bacillus* and *Paenibacillus* with inhibitory activity against *Ascosphaera apis*, an important bee fungal pathogen.
These results indicate that beneficial bacteria inhabiting the honeybee, especially honey stomach, can play important role not only in the AFB tolerance, but also in the resistance to other diseases, and therefore probiotic bacteria originating from the honeybee microbiota can be considered for probiotic use in their prevention.

29.5.2 Fur-Bearing Animals

For fur-bearing animals, probiotics are mostly used for the prevention of intestinal diseases that cause the highest loses at farms due to higher mortality, growth delay, and lower pelt quality. Pathogenic strains of *E. coli* and *Salmonella*, coccidiosis, and strongyloidosis belong to the most important causative agents of intestinal diseases (Süvegová et al. 1994). The inhibitive effect of probiotic enterococci against potentially pathogenic *E. coli* in foxes and minks was confirmed by Jørgensen (1988) and Gugolek et al. (2004). The positive effect of probiotics on the composition of digestive tract microbiota has a significant influence on the health status, and thereby also on the production.

Beneficial effects of probiotics on the morphology of the digestive tract mucosa and reduction of degenerative changes of the liver and kidneys can resulted in higher BWG and improvement of other production parameters (Gugolek et al. 2004). Higher BWG in foxes and minks after application of probiotics were reported by many authors (Gugolek et al. 2004; Balakiriev et al. 1994; Tauson 1984). Lorek et al. (2001) noted higher utilization of carbohydrates and gross energy from the diet in arctic foxes fed a diet containing *E. faecium* and *L. acidophilus*.

While the color type in fur animals is determined genetically, the fur color purity depends on different factors, including diet. Gugolek et al. (2004) observed that a mixture of *E. faecium* and *L. acidophilus* added to the diet of arctic foxes significantly improved a fur quality as compared with the group without addition of probiotics. Better fur quality was also observed in minks receiving probiotic *E. faecium* (Balakiriev et al. 1994).

Süvegová et al. (1994) confirmed a positive effect of a probiotic preparation containing *B. subtilis* and streptococci on the weight of newborn coypus and their growth in the first 2 months of life and during weaning. Probiotics were applied to mates during the second half of gravidity and to young during 4 months. Tauson (1984) studied the effect of addition of LAB on the reproductive performance in minks and blue foxes. Probiotic LAB have increased the number of newborns per mother and decreased mortality of the young animals.

29.5.3 Rabbits

Rabbit breeding for meat production has a great economic impact, above all due to high quality of meat—low cholesterol and total lipids and high content of protein. Intestinal diseases caused by *E. coli*, *Clostridium* sp., or *Eimeria* sp. are among the most important health problems at rabbit farms (Grobner 1982). Since lactobacilli, bifidobacteria, lactococci, and pediococci are not common inhabitants of digestive tract of rabbits, enterococci and yeasts are mostly used for probiotic purposes (Straw 1988; Yu and Tsen 1993). Enterococci are commonly present in the intestine of rabbits in sufficient numbers, 10³–10⁵ CFU/g of feces (Simonová and Lauková 2004). Simonová et al. (2005) isolated enterococci from feces of rabbits. Most of isolates showed good survival in the conditions of digestive tract (5% oxgall, pH 3), urease activity, and production of lactic acid. The majority of isolates were characterized as *E. faecium*, others as *E. faecalis*. Moreover, the isolates of *E. faecium* possessed bacteriocinogenic activity (Simonová and Lauková 2004). Bacteriocin-producing strain *E. faecium* with confirmed probiotic properties were administered to growing
rabbits (35 days of age) for 3 weeks. The inhibitory effect of *E. faecium* was noted by decreased numbers of *E. coli*, *Clostridium*-like sp., and coagulase-negative staphylococci in cecal contents. The numbers of *Eimeria* sp. oocysts was also reduced. Immunostimulative effect was confirmed by increased phagocytic activity (Szabóová et al. 2008). Bacteriocins produced by *E. faecium* strains, isolated from feces of rabbits, have shown ability to inhibit growth of *L. monocytogens*, *L. innocua*, and staphylococci (Simonová and Lauková 2007). Abdel-Samee (1995) found out that supplementation of probiotics to heat-stressed rabbits at a farm in Egypt significantly increased daily weight gains and improved reproductive parameters (litter size at weaning, weight at birth and at weaning). The incidence of diseases and mortality was also reduced as compared with control rabbits without addition of probiotics. Positive effect of probiotics on the nutrient digestibility, feed efficiency, and daily weight gains in weanling rabbits was reported by Yamani et al. (1992). Supuková et al. (2010) fed a combination of *L. fermentum*, *E. faecium*, maltodextrin, and FOS to rabbits. This combination significantly decreased number of intestinal disorders and increased daily weight gains.

### 29.5.4 Horses

Probiotics in equine practice are used for the prevention of intestinal disorders, for the substitution of beneficial microbiota after or during antibiotic therapy, when horses are stressed, and often also for the prevention or therapy of reproductive problems in mares (Moates 2009).

The horse cecum contains beneficial microorganisms that play an important role in the digestion of food. If its balance is impaired, the horse may have problems with nutrient absorption, which can result in the health disorders such as diarrhea or skin and hoof problems.

The addition of yeasts into the diet of horses can enhance the nutrient utilization from food because they are considered to stimulate the growth of beneficial bacteria living in the horse gut (Moates 2009). Glade and Biesik (1986) observed increased retention of nitrogen from a food supplemented with *S. cerevisiae*. Other authors also reported positive effects of yeasts on the nutrient digestibility—Glade (1991) in mares, Pagan (1990) in mature horses, and Hill and Gutsell (1998) in riding horses. Supplementation with *S. cerevisiae* reduces pH values, increases acetate concentration and numbers of cellulolytic bacteria in the cecum and colon, and in this way helps horses better tolerate a high-starch diet without developing acidosis and other digestive disorders (Moore and Newman 1994). Better utilization of nutrients has influence on milk quality. Glade (1991) found out that yeasts improved the quality of milk in mares and thereby also the growth of foals.

Some studies were performed with the per-oral application of probiotic LAB to horses, but their results were not very promising. Addition of a mixture of lactobacilli, *B. bifidum*, and *E. faecium* into the diet of mature horses had only limited influence on the nutrient digestibility of fat and certain minerals was increased. Supplementation of this mixture did not demonstrate a reduction of the risk of digestive disorders, such as acidosis, caused by feeding high-starch concentrates to horses (Swyers et al. 2008). *L. pensosus* strain WE7, which showed *in vitro* antagonistic activity against *E. coli*, *Streptococcus zooepidemicus*, *C. difficile*, and *C. perfringens* (Weese et al. 2004), was subsequently administered to neonatal foals. Surprisingly, foals receiving probiotics were experiencing higher incidence of diarrhea and other clinical disorders (e.g., colic, anorexia, weakness) in comparison with control animals Weese and Rousseau (2005). LGG, successfully used in animal species, was not able to transiently colonize the horse digestive tract (Weese et al. 2003).

On the other hand, LAB, especially lactobacilli, have with success been used in the prevention of reproductive problems in mares because they play an important role in the regulation of the vaginal microbiota. Fraga et al. (2008) isolated lactobacilli and enterococci from the vaginal
wall of mares. *L. mucosae, L. equi,* and *E. faecalis* have shown the highest antimicrobial activity against reproductive pathogens—*E. coli* and *S. aureus*. Morvayová et al. (2008) also isolated lactobacilli from the vagina of healthy mares and tested their inhibition effect against pathogenic bacteria received from the mare with chronic endometritis—*E. coli, S. aureus,* and *S. agalactiae*. Only one of the isolated strains was able to inhibit the growth of *E. coli*. In equine practice, lactobacilli have been successfully used for the therapy of *Candida* infections in mares (Hura 2008, personal communication). Rohrbach et al. (2007) received promising results with the treatment of intravenously administered *Propionibacterium acnes* to mares with persistent endometritis. Results indicated that application of propionibacteria improved successful conception of mares and the number of live delivered foals.

29.6 Conclusion

Probiotics represent an effective alternative to synthetic substances in disease prevention and growth performance promotion, although it is challenging to achieve comparable efficacy. In pigs, specific probiotics can be effectively used to support the development of a stable microbiota, to stimulate the immune system, and to prevent diarrheal diseases in neonatal piglets. During the weaning period, probiotics are effective in preventing PWD and stimulating growth. Probiotics in cattle have a wide application after birth, lactation, and fattening, or as pathogen inhibitors during conservation of silage. Probiotics may support the profitability and safety of poultry production and offer meaningful alternatives for natural and organic production. Research indicates that the application of viable microorganisms to poultry feed under certain conditions can improve animal health, increase their productivity, and improve quality of the production. For the probiotics to represent a real and effective alternative to antibiotics and chemotherapeutics, it is absolutely necessary to ensure their consistent high efficacy. The application of modern molecular genetic techniques is needed for better understanding molecular interaction of probiotics with endogenous microbial communities of the host. The trend for the future could focus on basic research to identify and characterize existing probiotic strains, define the precise modes of action, determine optimal doses needed for specific applications, assess their stability through processing, and search for possibilities of improving the effectiveness of probiotics by their combination with substances of natural origin. The use of potentiated probiotics may result in more effective modulation of the gastrointestinal ecosystem for improvement of animal health and growth. Probiotics not only beneficially affect animals but also protect human health. This observation has increased the emphasis placed on disease prevention as a means of reducing the use of antibiotics and reducing public fears about antibiotic residues in milk, meat, and other products of animal origin.

Acknowledgments

The work was supported by project NFP26220120109, project NFP26220220244 (Agency of the Ministry of Education, Science, Research and Sport of the Slovak Republic for the Structural Funds of the EU), by grant VEGA 1/0372/10 (Scientific Grant Agency of the Ministry of Education, Science, Research and Sport of the Slovak Republic and the Academy of Sciences), and by project SK0021, co-financed through the EEA financial mechanism, the Norwegian financial mechanism, and the state budget of the Slovak Republic.
References


Probiotics for Farm Animals


Chapter 30

Health Effects of Nonviable Probiotics

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30.1 Probiotics—Viable by Definition

According to the currently most popular definition of probiotics, issued by the Joint Food and Agriculture Organization/World Health Organization Working Group (Joint FAO/WHO Working Group Report on Drafting for the Evaluation of Probiotics in Food 2002), probiotics are “live microorganisms that, when administered in adequate amounts, confer a health benefit on the host.” Thereby, viability is an essential requirement for probiotics and, strictly speaking, inactivated probiotics as well as isolated components and metabolites of probiotics fall outside the current definitions of probiotics. Nevertheless, in this review, in the lack of better terminology, we will refer to inactivated probiotics as well as isolated components and metabolites of probiotics as nonviable probiotics, while acknowledging the inherent self-contradiction of this term. In this chapter, the role of viability in the proposed health effects of probiotics is discussed by reviewing the literature on the mechanisms of the health effects with special focus on the requirement of cell viability, and by comparing the health efficacy documentation of nonviable probiotics with that of viable probiotics.

30.2 Mechanisms of Probiotic Health Effects and Role of Viability

A major issue in the establishment of the clinical health effects of probiotic bacteria is the demonstration of the mechanisms of their beneficial outcomes. This is a challenging task, and in most cases, the mechanism of probiotic action remains to be fully understood. Owing to the complexity of the intestinal ecosystem and its interactions with the host, it is possible or even likely that multiple mechanisms are involved in most of the host–microbial interactions of probiotic bacteria. Identification of the multiple factors attributing to the homeostasis of a healthy microbiota is required before the underlying mechanisms can be explained.

30.3 Adhesion to Intestinal Mucus and Epithelial Cells

Adhesion to host tissues is thought to be one of the key mechanisms facilitating host–microbial interactions, such as the effects of microbes on the immune system of the host. Bacteria attached to the intestinal cell wall are far more likely to get in contact with host cells than bacteria in the intestinal lumen. The effect of cell viability on the mucus adhesion has been studied to some extent in vitro. It has been reported that viable and nonviable lactobacilli are equally adherent to intestinal mucus (Hood and Zottola 1988). Interestingly, the adherence may be dependent on the way by which the cells have been killed. Tuomola and others (2000) demonstrated that heat killing and protease treatments of Lactobacillus rhamnosus GG (LGG) and L. acidophilus LA1 were detrimental to the ability of these strains to adhere to human mucus, but other means of cell killing had no effect. Ouwehand and others (2000) showed that the effects of inactivation are strain dependent, and certain inactivation methods decrease the adhesion properties, whereas other inactivation methods may lead to increased adhesion. It is possible that cell wall structure, but not cell viability, determines the adhesion properties, and that different inactivation methods have different effects on the cell wall components. Heating in particular may have strong effects on the conformation of the cell wall components such as proteins. Maldonado Galdeano and Perdigón (2004) reported that heat killing of administered lactobacilli affects the localization of
the cells in the intestine. Viable bacteria were reported to be located in Peyer’s patches and lamina propria 10 min after administration to mice, whereas most heat-killed bacteria were located in the lumen and were rapidly cleared. It should be noted that while adhesion to host tissues as such may be equal between viable and nonviable probiotics, if viable probiotics are able to proliferate or temporarily colonize the host intestinal tissues, the duration of their adhesion in vivo may be longer than that of nonviable probiotics. In conclusion, viability does not appear to be a requirement for adherence, but it is clear that probiotic cells need to be viable if temporary colonization for a significant time is to be achieved. Thus, it may be assumed that viable probiotics are more likely to have an effect.

30.4 Inhibition of Growth and Adhesion of Pathogens
Many probiotic bacteria have been claimed to inhibit the viability or the adhesion of pathogenic bacteria in the intestine. When the antipathogenic activity is based on the production of antimicrobial substances in the intestine, it seems logical to assume that only viable and metabolically active cells are capable of such activity. In some cases, the antimicrobial substances may be already produced during the fermentation step of the product; in such case the presence and the stability of the antimicrobial metabolite is more important than the presence or the viability status of the probiotic. Some preclinical evidence exists for the efficacy of nonviable probiotics to reduce the adhesion of pathogens. For example, heat-killed cells of *L. acidophilus* LB have been reported to inhibit pathogen adhesion to epithelial cells, possibly through steric hindrance (Coconnier et al. 1993; Chauviere et al. 1992). The mechanism of pathogen inhibition dictates whether nonviable probiotics have effects. When the mechanism is based on passive adhesion to mucosal binding sites and subsequent blocking of the pathogen adhesion, nonviable probiotics can be assumed to be effective, at least on short term. However, when the mechanism is based on active antimicrobial functions or when (temporary) colonization to the mucosa is required, it seems clear that viable probiotics are more effective.

30.5 Microbiota Modulation
Aberrant intestinal microbiota is thought to play a role in many diseases. Several studies have indicated that administration of probiotic strains can modulate the intestinal microbiota, and this is believed to play a role in certain proposed health benefits of probiotic bacteria. Changes in the intestinal microbiota induced by live probiotics have been demonstrated in subjects with aberrant gut microbiota, such as kidney disease patients (Hida et al. 1996) and low-birth-weight neonates (Agarwal et al. 2003), but the effect of viable and nonviable bacteria on the gut microbiota has rarely been compared in a clinical setting. Kirjavainen and others (2003) reported that the neither live nor heat-killed LGG had effect on the levels of bifidobacteria, lactobacilli and enterococci, *Bacteroides*, or clostridia in atopic infants. Taken together, the role of viability in the modulation of gut microbiota is currently unclear and has not been sufficiently assessed in clinical trials. However, it appears that the effects of both viable and nonviable probiotics on the gut luminal microbiota in general are modest and temporary, and primarily affect only selected parts of the intestinal microbiota. These effects are typically measured from fecal samples, but in the intestine, the modulation of the mucosal microbiota may be far more relevant to host health than the modulation of the luminal or fecal microbiota.
30.6 Intestinal Permeability

Compromised mucosal barrier and increased gut permeability are typical attributes of several gastrointestinal disorders, and it has been suggested that the restoration and stabilization of the gut mucosal barrier may have a role in the mechanisms of the proposed health effects of probiotics (Salminen et al. 1996). Improvement of gut barrier function by administration of probiotic bacteria has been demonstrated in animal models and in humans with varying success. The efficacy appears to be strain specific but is also dependent on the condition of the host. The current evidence for the role of viability in the improvement of intestinal permeability by probiotic bacteria is still limited. Most studies have been performed using live probiotics. The few exceptions include the work by Montalto and others (2004), who reported that inactivated *L. acidophilus* LB is capable of protecting tight junctions from aspirin-induced damage. On the other hand, Yan and Polk (2002) demonstrated improved protection of intestinal epithelial cells from apoptosis using live LGG or cell-free supernatant, but a similar effect was not observed for heat-killed bacteria. Isolauri and others (1991) found no difference in the intestinal permeability of children with acute diarrhea receiving either viable LGG or nonviable yogurt bacteria. Heat-killed cells of *L. acidophilus* LB have been reported to inhibit cell invasion by pathogens (Coconnier et al. 1993). The cell-free supernatant of *L. acidophilus* LB has also been shown to protect against brush border damage of intestinal cells (Lievin-Le Moal et al. 2002). Secreted products of certain probiotic bacteria have been suggested to induce colonic mucin secretion (Caballero-Franco et al. 2007). In mice, both viable and heat-killed lactobacilli have been reported to increase the resistance against parasites (Bautista-Garfias et al. 2001). Resta-Lenert and Barret (2003) reported that only viable probiotics were able to protect human intestinal epithelial cells from damages caused by enteroinvasive *Escherichia coli*. Gotteland and others (2001) compared viable and heat-killed LGG, and reported that neither had an effect on intestinal mucosal barrier, but viable LGG reduced the alteration of gastric mucosal barrier resulting from indometacin treatment. The current limited evidence suggests that, in general, viable probiotics appear to be more effective in the enhancement of gut barrier function. This was recently highlighted in a study linking the in situ metabolite production (in this case the production of acetate by bifidobacteria) with the protective effect of probiotics (Fukuda et al. 2011). Nevertheless, in certain cases, nonviable probiotics may also be effective.

30.7 Regulation of Immune System and Function

The intestinal immune system is the largest and most complex part of the human immune system (Mowat 2003). A number of studies have shown that probiotic bacteria are able to modulate immune system and function, including both innate and acquired immune responses. Experimental studies assessing the effects of nonviable probiotic bacteria and their cell components on the immune system are numerous, although clinical evidence is limited. Examples of clinical effects proposed for nonviable *Lactobacillus* probiotics include enhancement of salivary IgA production in elderly volunteers (Kotani et al. 2010) and modulation of T-cell responses in healthy adults (Hirose et al. 2006). An elegant study by van Baarlen and co-workers (2009) demonstrated in a clinical setting that not only the viability but also the growth phase of the cells affects the immunomodulatory effects of probiotics. By applying biological pathway reconstruction, the authors showed that the host mucosal responses to dead and stationary phase probiotics were more similar to each other but different from the response to bacteria from the mid-logarithmic phase.
The role of probiotic viability in immune modulation is dependent on the mechanism of action. If immune modulation is mediated by individual cell compounds, it is possible that viability as such is not a factor since dead cells may also exhibit similar cell compounds. On the other hand, when the immune effects are mediated by locally produced metabolites, it is clear that viability is required. Preclinical evidence exists for the involvement of both isolated cell components and metabolites produced by the cells on immune modulation. Probiotic cell components associated with in vitro immunomodulatory properties include cell wall extracts (Solis Pereyra and Lemonnier 1993), lipoteichoic acids (Matsuguchi et al. 2003), bacterial DNA (Rachmilewitz et al. 2002; Takahashi et al. 2006), and S-layer proteins (Konstantinov et al. 2008).

Certain in vitro studies have directly compared the effects on innate immunity of viable and inactivated probiotics, and often both viable and nonviable probiotics have been equally effective. Haller and co-workers (2000) reported that both viable and heat-killed lactobacilli induce the proliferation of human natural killer cells in equal manner. Korhonen and co-workers (2001) reported that viable and heat-killed LGG induced nitric oxide production by macrophages, and suggested that lipoteichoic acid is the active component of LGG. Perdigón and co-workers (1986) found that viable and nonviable probiotics were equally effective in increasing the macrophage enzyme activity and the phagocytic activity in mice. Dehlink and co-workers (2007) studied the effects of probiotics on cytokine production in an intestinal cell–leukocyte co-culture model, and reported that viable probiotics were slightly but nonsignificantly more potent inducers of cytokine production than nonviable control probiotics. Gill and Rutherford (2001) reported that viable and killed cells of Bifidobacterium lactis HN019 were able to enhance cell phagocytic responses in mice peripheral blood cells, but only viable cells increased the phagocytic activity of peritoneal cells. Some studies have suggested that viable cells are more effective in the modulation of innate immunity. Miettinen and others (1996) studied the induction of cytokine production of human blood mononuclear cells by both viable and nonviable bacteria. Viable probiotics were found to be more potent inducers of tumor necrosis factor α (TNF-α) production than the dead probiotics. Lee and Lee (2005) suggested that viable bacteria are required for the enhancement of ex vivo phagocytic activity of murine peritoneal leukocytes. Ma and others (2004) concluded that only viable Lactobacillus reuteri has a potent anti-inflammatory activity by inhibiting TNF-α-induced production of IL-8. Conversely, Zhang and others (2005) demonstrated that both viable and heat-killed LGG had anti-inflammatory effects, although high doses of viable LGG resulted in pro-inflammatory effects (increased IL-8 production).

In the case of adaptive immune responses, studies comparing the effects of viable and nonviable probiotics head-to-head have mostly been in the favor of viable probiotics. Both viable and heat-inactivated yogurt bacteria have been shown to increase the amount of B-lymphocytes in mice, but viable bacteria had stronger effect (De Simone et al. 1987). No differences in T-cell proliferative responses were observed. Perdigón and others (1995) reported that viable but not nonviable L. casei inhibited Salmonella infection in mice. The protective effect of viable probiotics was attributed to specific IgA production, although the effects of nonviable probiotics on IgA production were not assessed. Later, Maldonado Galdeano and Perdigón (2004) presented evidence that viability of the probiotics affects the stimulation of mucosal immune system. Viable probiotics induced the number of cytokine- and IgA-producing cells in the intestinal mucosa to higher extent and for longer periods than did the nonviable probiotics. Similar results have been reported in infants with rotavirus diarrhea (Kaila et al. 1995; Majamaa et al. 1995). Moreover, viability was shown to be a requirement for increased mucosal antibody response to cholera toxin (Gill and Rutherfur 2001). However, Mohamadzadeh and others (2005) demonstrated that both viable and killed Lactobacillus cells are able modulate the phenotype and functions of human myeloid
dendritic cells. Colonization may be necessary for certain adaptive immune responses, at least in murine models. Ibnou-Zekri and others (2003) demonstrated that while both colonizing and noncolonizing strains of *Lactobacillus* were able to activate mucosal B-cell responses, the immune responses were different between the two strains, and only the colonizing strain increased the amount of *Lactobacillus*-specific IgA.

In conclusion, both viable and nonviable probiotic bacteria have been shown to modify immune responses. Several components of the cells have been linked with immunomodulatory effects. There is evidence suggesting that both viable and nonviable probiotics are capable of enhancing the innate immune function, but viable probiotics are more potent inducers of adaptive immune responses such as antibody production. The role of viability in the proposed mechanisms of probiotic action is summarized in Table 30.1. All properties of probiotics, viable or nonviable, are strain dependent.

### 30.8 Beneficial Effects of Probiotics and Role of Viability

Probiotic microbes have been linked with a range of beneficial effects on host health, some of which are well documented whereas others need further assessment. The diversity of the suggested health effects is remarkable. The target of most of the proposed health effects of probiotics is the gut, but there are also an increasing number of proposed health effects targeted outside the gastrointestinal tract. Here the proposed benefits of probiotics are reviewed, with a special focus on the role of the viability of the probiotics in the health effects.

### 30.9 Diarrhea

Numerous clinical studies aiming at the treatment or prevention of diarrheal diseases by use of probiotics have been published. Most studies have been conducted using *Lactobacillus* strains as probiotics, although other probiotics such as bifidobacteria and *Saccharomyces boulardii* have also been used. Efficacy of viable probiotics in shortening of the duration of rotavirus diarrhea bacteria is well established and has been reviewed in meta-analyses (Sazawal et al. 2006; Szajewska and Mrukowicz 2001;
Allen et al. 2004). Prevention of antibiotic-associated diarrhea with viable probiotics also appears (Kale-Pradhan et al. 2010). Recent meta-analyses of controlled clinical trials suggest that probiotics reduce the risk of acute diarrhea among both children and adults, while the attempts to prevent travellers’ diarrhea using probiotics have been only partly successful (Sazawal et al. 2006). Prevention of *Clostridium difficile*–associated diarrhea by probiotic bacteria is also promising (McFarland 2006).

Live bacteria have been used in most studies on prevention and treatment of diarrhea with probiotics, and comparisons with nonviable bacteria are rare. The efficacy of heat-killed *L. acidophilus* LB in the treatment of acute diarrhea has been investigated (Simakachorn et al. 2000), and has even been proposed to be more effective in the treatment of chronic diarrhea than a viable nonspecified strain of *L. acidophilus* (Xiao et al. 2003). The importance of viability in probiotic treatment of rotavirus diarrhea has been assessed in a small-scale clinical trial (Kaila et al. 1995). The authors reported no difference in the duration of diarrhea between the two study groups receiving either viable or heat-killed LGG. The study setting did not include a proper placebo group, thus it is impossible to evaluate whether the administered probiotics had a shortening effect on diarrhea, but the duration of diarrhea was short in both groups. There was no difference between the groups in the concentration of immunoglobulin-secreting cells in serum at the acute stage of diarrhea, but at convalescent stage, the subjects receiving viable probiotics had higher levels of rotavirus-specific antibody-secreting cells. Taken together, there is some evidence suggesting that nonviable probiotics are effective in the treatment of diarrhea, but the vast majority of the current clinical trials have been conducted using live probiotics, and thus there is more scientific evidence for the efficacy of viable probiotics than for the nonviable probiotics. Earlier, Ouwehand and Salminen (1998) concluded that both viable and nonviable probiotics are useful for short-term treatment or prophylactic treatment of diarrhea, but viable probiotics are necessary for enhanced immunological response.

### 30.10 Irritable Bowel Syndrome and Constipation

Probiotics are mainly consumed by the general population, free of serious underlying diseases. Despite being generally healthy, many healthy subjects experience occasional disturbances in their bowel habits. A specifically important subgroup of the general healthy population are the subjects who have irritable bowel syndrome (IBS), a gastrointestinal condition producing symptoms such as abdominal pain, flatulence, variable bowel habit, and bloating, without an obvious cause. Many successful trials on the management of IBS by viable probiotics have been published (Aragon et al. 2010). While the majority of clinical trials on the effects of probiotic therapies on the treatment of IBS have been carried out using live probiotic strains, in one blinded study heat-inactivated cells were used as controls for viable cells (Tsuchiya et al. 2004). The administration of the viable product resulted in subjective improvement of the symptoms in 80% of the patients, compared with 40% in the control group, suggesting that viable probiotics may have stronger effect in the treatment of IBS. Probiotics have been studied to some extent for their efficacy in relieving constipation; however, the effect of viability of probiotic bacteria in the treatment or prevention of constipation has not been assessed to date.

### 30.11 Inflammatory Bowel Disease

Inflammatory bowel disease (IBD) is a common name given to a group of chronic gastrointestinal disorders, including Crohn’s disease, ulcerative colitis, and pouchitis. The intestinal microbiota is
thought to have a role in the pathogenesis of IBD (Shanahan 2002). Probiotic bacteria have been proposed as therapeutic agents for the treatment of IBD (Gionchetti et al. 2002). Studies investigating the effects of viable probiotics in the treatment of Crohn’s disease have yielded inconsistent results. Nonviable probiotics for Crohn’s disease are yet to be tested in a clinical setting. Studies on the efficacy of certain viable probiotics on ulcerative colitis and pouchitis are limited but encouraging—again, clinical trials with nonviable probiotics are missing.

While the role of probiotic viability in the treatment of IBD cannot be evaluated based on clinical trials, in certain studies this aspect has been assessed using in vitro and murine models. A study using a murine colitis model suggested that live bacteria are not required for the protective effects of probiotics, as dead bacteria are as effective as live bacteria in attenuating the severity of colitis (Rachmilewitz et al. 2004). The mechanism of the amelioration of experimental colitis was attributed to DNA of probiotic bacteria. The results of another study (Sturm et al. 2005) suggested that the beneficial effect of *E. coli* Nissle 1917 is attributed to factors secreted by the cell into the surrounding medium. On the other hand, results from a study using an experimental murine colitis model (Castagliuolo et al. 2005) suggested that live bacteria in adequate amounts are required for the protective effects to occur. In conclusion, some experimental data suggest that viability may not be necessary for the suggested health benefits, but the results from the animal studies are inconclusive and the potential efficacy of nonviable probiotics is yet to be confirmed in a clinical trial.

### 30.12 *Helicobacter pylori* Eradication

Attempts to use probiotic bacteria or their culture supernatants in the eradication of *Helicobacter pylori* have yielded somewhat conflicting evidence. While full eradication of *H. pylori* by probiotics appears unlikely, probiotics may help in improving the eradication rates or suppress the adverse intestinal effects of antibiotic treatments targeted against *H. pylori* (Zou et al. 2009; please also see Chapter 23). Both viable (Zou et al. 2009) and nonviable (Canducci et al. 2000) probiotics have been reported to increase the eradication rates of a standard anti-*H. pylori* regimen. Studies by de Vrese and Schrezenmeir (2002) suggested that both viable and nonviable probiotics are equally effective in the treatment of *H. pylori* infections. On the other hand, Cats and others (2003) concluded that viability is required for the inhibitory activity in vitro. Similarly, Cruchet and others (2003) observed a small but statistically significant reduction in *H. pylori* infection in children consuming viable probiotic bacteria but not in children consuming nonviable probiotics. In an in vitro study, Rokka and co-workers (2008) compared the effects of live or heat-killed preparations of LGG, *L. plantarum* MLBPL1, and an unnamed strain of *Lactococcus lactis* on the adhesion of *H. pylori* on gastric adenocarcinoma cells and the *Helicobacter*-induced IL-8 production. Live lactic acid bacteria significantly inhibited the adhesion of *H. pylori*, but heat-killed strains had a nonsignificant effect. However, both live and heat-killed strains prevented the increase in IL-8 production, with the live probiotics being more effective. Thus, the current evidence for the role of viability of probiotic bacteria in the treatment of *H. pylori* infections is somewhat conflicting; however, overall at present the strength of the evidence appears to be far stronger for viable probiotics. It is possible that the requirement for viability is strain specific. Alternatively, several mechanisms may be involved, and these may be different for viable and nonviable probiotics. The effects of viable and nonviable probiotics in the treatment and prevention of intestinal disorders are summarized in Table 30.2.
30.13 Bacterial Vaginosis and Candidiasis

Bacterial vaginosis is a disease in which the lactobacilli microbiota of the vagina is replaced by other bacteria, typically gram-negative anaerobes. Successful treatment of bacterial vaginosis by live probiotic lactobacilli has been reported (Reid et al. 2001; Reid et al. 2003), although not all studies have yielded positive results (Eriksson et al. 2005). As always with probiotics, the beneficial effects are strain specific. The role of probiotic viability has not been assessed in the prevention or treatment of bacterial vaginosis. However, it has been demonstrated that for the prevention of urinary tract infections, colonization of the probiotic strains is critical. Furthermore, although the mechanism of the probiotic action is currently unclear, it has been suggested to involve the production of antimicrobial compounds by colonized bacteria. These considerations suggest that viability of the bacteria is a requirement for the beneficial effects of probiotics in the prevention and treatment of urogenital tract infections.

Opportunistic fungal infections such as candidiasis are increasingly common and serious problems in immunocompromised subjects. Both viable (Wagner et al. 1997) and inactivated (Satonaka et al. 1996; Wagner et al. 2000) probiotic bacteria have been shown to be effective in the prevention of candidiasis in experimental murine models. Currently, human clinical evidence is lacking, but if effective, nonviable probiotics could be an interesting alternative for immunocompromised subjects such as AIDS patients. At present there is little evidence for the efficacy of probiotic treatments in vaginal infections caused by Candida yeasts.
30.14 Lactose Digestion

With the exception of the populations of Northern European origin, lactose maldigestion resulting from lactase deficiency affects the majority of adults worldwide. Lactose maldigesters tolerate yogurt usually better than nonfermented dairy products, owing to the lactase activity of the yogurt starter cultures (Kolars et al. 1984). However, it is important to remember that strain-specific differences in lactase activity may vary 100-fold, even between the strains used normally in yogurt manufacture (Sanders et al. 1996). Not all probiotic strains are able to ferment lactose or alleviate lactose intolerance, and the effect is stronger in fermented products than in nonfermented products. Lactase production is strain dependent, but also depends strongly on the growth conditions of the bacteria, for example, the presence of lactose (Jiang et al. 1996). This may explain why yogurt bacteria are able to degrade lactose from the yogurt itself, but fail to have an effect on the additional lactose consumed together with the meal (Martini et al. 1991). The ability of certain strains of lactic acid bacteria and bifidobacteria to improve lactose digestion is well documented. In addition to bacterial lactase activity, the mechanisms are believed to involve delayed gastrointestinal transit, improvement of the functions of the commensal microbiota, and reduced sensitivity to symptoms (de Vrese et al. 2001).

Most studies comparing the efficacy of live and dead probiotics in improving lactose digestion have been performed using traditional yogurt bacteria, not probiotics. Currently, the majority of the clinical studies comparing viable and pasteurized yogurt suggest that live cells are more effective in improving lactose digestion (Lerebours et al. 1989; Pelletier et al. 2001). However, viability as such may not be the factor responsible for the superiority of nonpasteurized yogurt over pasteurized yogurt. de Vrese and others (2001) compared the effect of yogurt containing either live bacteria or dead bacteria. They concluded that to improve lactose digestion, the bacteria need not be alive, but intact cell walls are required to protect the active β-galactosidase during gastrointestinal passage. The beneficial effect of pasteurized bacteria was low, but the effect of bacteria killed with gamma irradiation was similar to the effect of viable bacteria. Following ingestion, yogurt bacteria are not able to degrade dietary lactose derived from other sources than the yogurt itself (Martini et al. 1991), suggesting that the metabolic activity of these cells in the intestine is low or that the cells, if remaining alive, utilize preferably other sources of energy.

30.15 Allergic Diseases
30.15.1 Prevention of Allergies

An increasing amount of evidence suggests that selected live probiotic strains may be effective in prevention of allergic diseases. In particular, prevention of atopic eczema by probiotic treatment to pregnant mothers and newborn infants seems effective (Tang et al. 2010), although for some strains the clinical trials have yielded conflicting results (Boyle and Tång 2006). In addition to prevention, the management of atopic eczema by probiotic treatments has also been proposed; however, in general, it appears that the treatment of allergies by probiotics is not as effective as prevention. Almost all of the clinical trials assessing the efficacy of probiotics against allergies have been carried out with live probiotics. In one small trial, the efficacy of viable and heat-inactivated LGG was compared in the management of atopic disease in infants (Kirjavainen et al. 2003). While both of the treatments as well as the placebo treatment appeared to improve the allergic symptoms, the administration of heat-killed LGG was attributed to adverse side effects such as diarrhea. The underlying mechanism for the adverse effects was unknown. However, adverse
events appeared to be more frequent among infants who had high numbers of clostridia and/or *Bacteroides* in feces before treatment administration, and by chance these bacterial groups are more frequent in the nonviable group than in the viable group at baseline—it is therefore difficult to judge whether the gastrointestinal symptoms were actually related to the treatment allocation or to the initial composition of the microbiota of the infants. Van de Water and others (1999) reported fewer subjective allergy symptoms in subjects consuming yogurt containing viable bacteria, compared with subjects consuming heat-inactivated yogurt. These two reports suggest that viable bacteria are the preferred choice for the management of allergic diseases. However, certain reports have suggested that both viable and nonviable probiotics may be useful in the treatment of allergic rhinitis. Peng and Hsu (2005) compared the effects of viable and heat-killed *L. paracasei* in the treatment of house dust mite–induced perennial allergic rhinitis, and concluded that the two therapies were equally effective compared to placebo. Two reports assessing the effect of viable (Xiao et al. 2006) and heat-killed (Ishida et al. 2005) probiotics on the prevention of Japanese cedar pollen allergy yielded comparable results, with both studies suggesting a modest improvement in certain subjective symptoms. It is possible that the requirement for viability is more important in the management of eczema than in the management of allergic rhinitis.

### 30.16 Anticarcinogenic Effects

Possibly the most controversial of all health benefits attributed to probiotic bacteria is the anticancer activity. While the direct evidence for suppression of cancer in humans by probiotic bacteria is lacking, there is some indirect evidence, mostly from experimental laboratory trials and epidemiological studies. In regard to the role of probiotic viability, clinical evidence is limited, but both viable and nonviable probiotics have been used in some preliminary studies. Two early reports suggested that oral administration of *L. casei* Shirota reduces the risk of recurrence of superficial bladder cancer (Aso and Akazan 1992; Aso et al. 1995). Moreover, preparation of heat-killed *L. casei* Shirota has been used in phase II and phase III trials on treatment of carcinoma of the uterine cervix, resulting in a significant beneficial effect on tumor reduction (Okawa et al. 1989; Okawa et al. 1993). The efficacy of intrapleural instillation of the same preparation in the treatment of malignant pleural effusions secondary to lung cancer has been assessed in a phase III trial (Masuno et al. 1991); patients receiving heat-killed *L. casei* Shirota had significantly higher response to the treatment (73.7%) compared with the placebo group (39.5%). Prolonged survival in the probiotic group was also observed. Direct clinical comparisons between viable and nonviable probiotics in cancer prevention or treatment are lacking, but comparisons are limited to a number of preclinical studies. Viable but not heat-killed lactic acid bacteria have been suggested to have antigenotoxic effect on isolated colonic cells of rats (Pool-Zobel et al. 1996). However, in this study, soluble metabolites, extracted peptidoglycan, and ultrasonicated cells also had an effect, suggesting that viability as such was not the critical factor, but the beneficial effect was susceptible to heat. In a study by Kato and others (1994), viable *L. casei* was found to be more effective than heat-killed *L. casei* in the prevention of secondary tumors in preimmunized mice. On the other hand, heat-killed lactic acid bacteria are more effective than viable bacteria in the binding of aflatoxin, a potent dietary carcinogen (El-Nezami et al. 1998). Taken together, studies comparing the antitumor or anticarcinogenic properties of viable and nonviable probiotic bacteria are rather scarce. The efficacy of inactivated probiotics has been shown in some preclinical studies, suggesting that both viable and nonviable could potentially be effective.

The effects of viable and nonviable probiotics in the treatment and prevention of bacterial vaginosis, candidiasis, lactose digestion, allergies, and cancer are summarized in Table 30.3.
Preliminary data suggest that live probiotics may be useful in the treatment of short-bowel syndrome (SBS; please see Chapter 23). The treatment of SBS with probiotics appears very promising, but the safety of the treatments is a critical issue, as SBS patients may be more susceptible to bacterial translocation and bacteremia than other consumers of probiotics. Therefore, in this particular population, nonviable probiotics could offer an interesting alternative for viable probiotics. Currently, however, there are no studies comparing the efficacy of viable and nonviable probiotics in the treatment of SBS and the mechanisms behind the proposed beneficial effects of viable probiotics are unknown.

Necrotizing enterocolitis (NEC) is an abdominal emergency affecting some preterm neonates in neonatal intensive care units, which is thought to have a link with abnormal intestinal microbiota. Early reports on the efficacy of live probiotics against NEC have been promising, as reviewed in Chapter 23. As with SBS, in the case of NEC patients, the safety of the probiotic treatments needs to be considered since preterm infants have immature immune systems. Unfortunately, the role of bacterial viability in the treatment of NEC with probiotics and the potential benefits of nonviable probiotics have not been studied to date.

Certain bacterial species of the normal gut microbiota are capable of degrading oxalate in the gut, protecting the host from hyperoxaluria and the formation of calcium oxalate kidney stones. However, not all subjects have sufficient levels of oxalate degraders in their microbiota. Specific strains of the genus *Lactobacillus*, such as *L. acidophilus* NCFM, have been reported to be effective oxalate degraders.

### Table 30.3  Comparison of Health Effects of Viable and Nonviable Probiotics (Liver, Kidney, and Urogenital Tract Diseases; Candidiasis; Lactose Digestion; Allergies; and Cancer)

<table>
<thead>
<tr>
<th>Target</th>
<th>Viable</th>
<th>Nonviable</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacterial vaginosis</td>
<td>++</td>
<td>○</td>
<td>No data on nonviable</td>
</tr>
<tr>
<td>Candidiasis</td>
<td>(+)</td>
<td>(+)</td>
<td>Experimental data only</td>
</tr>
<tr>
<td>Lactose digestion</td>
<td>+++</td>
<td>– / (+)</td>
<td>Heat treatment detrimental to enzymes</td>
</tr>
<tr>
<td>Prevention of eczema</td>
<td>++</td>
<td>○</td>
<td>No data on nonviable</td>
</tr>
<tr>
<td>Treatment of eczema and/or food allergies</td>
<td>+, +/-</td>
<td>–</td>
<td>Nonviable may cause adverse effects</td>
</tr>
<tr>
<td>Treatment of respiratory allergies</td>
<td>+, +/-</td>
<td>+</td>
<td>Equal in the treatment of rhinitis?</td>
</tr>
<tr>
<td>Cancer: recurrence of bladder cancer</td>
<td>+</td>
<td>○</td>
<td>No data on nonviable</td>
</tr>
<tr>
<td>Cancer: tumor reduction</td>
<td>○, (+)</td>
<td>(+)</td>
<td>No clinical data on viable</td>
</tr>
<tr>
<td>Cancer: binding of carcinogens</td>
<td>+, (+)</td>
<td>(+)</td>
<td>Nonviable more effective in vitro</td>
</tr>
</tbody>
</table>

*Note:* ++++, Well documented; ++, appears effective; +, potential, needs further assessment; (+), experimental/in vitro evidence only; +/-, contradicting evidence; –, no effect; ○, no data available.

### 30.17 Other Proposed Health Benefits

Preliminary data suggest that live probiotics may be useful in the treatment of short-bowel syndrome (SBS; please see Chapter 23). The treatment of SBS with probiotics appears very promising, but the safety of the treatments is a critical issue, as SBS patients may be more susceptible to bacterial translocation and bacteremia than other consumers of probiotics. Therefore, in this particular population, nonviable probiotics could offer an interesting alternative for viable probiotics. Currently, however, there are no studies comparing the efficacy of viable and nonviable probiotics in the treatment of SBS and the mechanisms behind the proposed beneficial effects of viable probiotics are unknown.

Necrotizing enterocolitis (NEC) is an abdominal emergency affecting some preterm neonates in neonatal intensive care units, which is thought to have a link with abnormal intestinal microbiota. Early reports on the efficacy of live probiotics against NEC have been promising, as reviewed in Chapter 23. As with SBS, in the case of NEC patients, the safety of the probiotic treatments needs to be considered since preterm infants have immature immune systems. Unfortunately, the role of bacterial viability in the treatment of NEC with probiotics and the potential benefits of nonviable probiotics have not been studied to date.

Certain bacterial species of the normal gut microbiota are capable of degrading oxalate in the gut, protecting the host from hyperoxaluria and the formation of calcium oxalate kidney stones. However, not all subjects have sufficient levels of oxalate degraders in their microbiota. Specific strains of the genus *Lactobacillus*, such as *L. acidophilus* NCFM, have been reported to be effective oxalate degraders.
(Turroni et al. 2007). Small-scale open-label studies have also suggested that a combination of several probiotic strains may be effective in reducing the urinary excretion of oxalate (Campieri et al. 2001; Lieske et al. 2005). At this point, the use probiotics in the treatment of hyperoxaluria shows some promise, but randomized controlled trials are currently lacking. Role of probiotic viability in the prevention of hyperoxaluria has not been assessed. It seems obvious that if the mechanism of probiotic action is the metabolic degradation of oxalate, viability is a prerequisite for probiotic action.

It is now known that bacterial metabolites of intestinal microbiota, typically aberrant in dialysis patients, contribute significantly to the levels of uremic toxins in the blood. Restoration of the normal intestinal microbiota in patients with kidney disease by administration of probiotics, resulting in potential reduction of circulating uremic toxins, has been proposed. Hida and others (1996) observed reduced levels of uremic toxins and improved composition of fecal microbiota in renal disease patients receiving probiotics. Similarly, a significant reduction of uremic toxins in blood and improvement of nutritional state by L. acidophilus NCFM in dialysis patients was reported by Dunn and others (1998). Thus, preliminary data suggest that probiotic therapies may be useful in the treatment of end-stage renal disease, by reducing the levels of uremic toxins originating from aberrant gut microbiota. No data are available for the role of viability of the probiotics in the treatment of kidney disease. As the favored end point of the treatments is the normalization of gut microbiota, it appears reasonable to assume that viable probiotics are required to achieve this goal. Probiotics have also been proposed to have beneficial effects on oral health and in liver diseases. Despite some preclinical studies (Segawa et al. 2008; Tanzer et al. 2010), clinical data on the efficacy of nonviable probiotics in these health targets are currently lacking. Viable probiotics have been linked with reduced rates of upper respiratory tract infections in several clinical studies (Leyer et al. 2009; Vouloumanou et al. 2009). Nonviable probiotics have not been assessed in this respect, although some preclinical evidence exists for the efficacy of nonviable probiotics against chest infections (Villena et al. 2009).

30.18 Conclusions

As illustrated in this review, a significant number of health benefits have been proposed for the probiotic bacteria. In some cases, the scientific evidence for them is strong, whereas some of the suggested health benefits have hardly been tested in a clinical setting. Viability of probiotics is often considered a requirement for the beneficial health effects, and it is, by definition, a requirement for microbes to be considered as probiotics. However, the current review and an earlier review by Ouwehand and Salminen (1998) suggest that while viable probiotics appear to be more effective than nonviable bacteria in some situations, nonviable probiotics may also possess certain beneficial health effects. Examples of health targets in which nonviable probiotics could have a role include stimulation of the innate immune system, acute treatment of diarrhea, improvement of the success rates of H. pylori eradication therapies, and treatment of allergic rhinitis. That being said, the current evidence demonstrates that, in general, viable probiotics are more effective than nonviable probiotics and are therefore the preferred choice for most probiotic applications.

References


Chapter 31

Probiotics: Safety and Efficacy

Seppo Salminen and Atte von Wright

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31.1 Introduction

The definition of probiotics has evolved over a long time, and the history of probiotics is long-standing. Therefore, the exact definition has been at times difficult to reach. Probiotics have been mentioned as fermented health-promoting products during early history. Among the scientific references, Döderlein (1892) proposed the use of microorganisms for a specific medical condition,
such as vaginal infections. Some years later Dr. Tissier at the Pasteur Institute in France isolated a *Bifidobacterium* strain from a breast-fed infant (Tissier 1905), demonstrating that bifidobacteria are the most common organisms in the feces of breast-fed infants and today are thought a hallmark of the healthy microbiota of breast-fed infants. Elie Metchnikoff (1907) was among the first scientists who proposed the health benefits of and also reported health claims for members of healthy gut microbiota in his book *The Prolongation of Life*. Along the same path, in 1917 Alfred Nissle isolated an *Escherichia coli* that he used successfully to treat acute intestinal diseases. Thus, the suggestion for a health claim for probiotics is not a new one.

The current definition used also in the European Union (EU) is mainly based on earlier definitions and formulated by the International Life Sciences Institute Europe and the World Health Organization (WHO) (Salminen et al. 1998; WHO 2001, 2002). The WHO working group definition of probiotics concludes that probiotics are “live microorganisms, which when administered in adequate amounts, confer a health benefit on the host” (WHO/FAO 2002). This differs from the definition used often in Asia, where the term probiotic often covers both live microorganisms and also nonviable cells of probiotics, when a health benefit has been demonstrated (Salminen et al. 1999).

The viability of probiotic microorganisms is especially emphasized in the present definitions. Viability has often been interpreted as synonymous with culturability. However, the emergence of the fact that microorganisms can be in a physiological state that does not allow multiplication but leaves them viable (although not cultivable) may require rethinking of the definitions. It is also well known that the majority of intestinal microorganisms are certainly viable and multiply in the gut but cannot be grown in standard laboratory conditions. Therefore probiotics can be viable without having to be culturable. Because of these facts, the term viability needs to be redefined as well in the future.

As the area is very difficult to comprehensively define, we have taken the terminology approach to clarify the approaches to the safety aspects of probiotics and the health claims. We also decided to define the procedures starting from probiotics and proceeding from characterization to health claims. The unified approach takes different points of view to terminology to clarify the basis and meaning of terms related to probiotics and health claims. This approach also facilitates the clarification of the road from characterizing probiotics and their origin and leading to the human health claims.

### 31.2 Characterization of Probiotic Bacteria

An up-to-date identification and classification of microorganisms constitute the starting point for the assessment of microbial properties. A reliable identification by accurate and modern methods should confirm the identity of each strain in commercial use. Such identification is also necessary for proper labeling of probiotic products. An accurate and correct identification allows linking the microorganism to what is already known about the corresponding microbial group, permitting the prediction of some of its properties as described in the safety and efficacy assessment parts (Section 31.3).

Recently, molecular techniques have replaced or complemented most traditional phenotypic methods. DNA–DNA hybridization is the current gold standard for determination of bacterial identification, with two strains being considered to belong to the same species if their DNA–DNA relatedness is ≥70%. Phylogenetically based approaches, such as sequence analysis of the 16S rRNA gene, are currently the most commonly used methods for bacterial species identification in the food industry. In general, microorganisms sharing a 16S rRNA gene homology that is higher than 97% are considered members of the same species. An EU-funded recent research project (Prosafe) concluded that biochemical tests should not be used as stand-alone methods for identification of probiotics. It was recommended that the use of 16S rRNA gene sequence analysis
should be an appropriate tool for routine species identification on the basis of its high reproducibility and data exchangeability (Vankerckhoven et al. 2008). The FAO-WHO expert group on probiotics recommends that phenotypic tests should be done first, followed by genetic identification, using methods such as DNA–DNA hybridization, 16S rRNA sequence analysis, or other well established methods (FAO 2006).

Establishing the true identity of a microorganism is an important first step for the assessment of its safety and efficacy. For proper species identification, it is extremely important to consider that fact that probiotic effects are strain specific and thus it is necessary to identify the microorganisms at the strain level.

**31.3 Safety Aspects of Probiotics**

Fermented foods containing live microorganisms have been consumed for thousands of years worldwide. Spontaneous food fermentations were initially used to preserve foods by inhibiting the growth of spoilage/pathogenic microorganisms. A number of different species of lactic acid bacteria (LAB) have been used in food fermentation with the aim of increasing the shelf life of foods and to improve food safety. Only recently, the first target has been to improve consumer health. This has lead to the redefinition of the basis of the probiotic concept.

Currently used common probiotics, mainly from the genera *Lactobacillus* and *Bifidobacterium*, have not shown any pathogenicity traits. With the exception of enterococci, LAB and bifidobacteria are rarely involved in infection. Nevertheless, some rare cases of infection have been reported, and they emphasize the characterization of probiotic strains and also the preclinical assessment of probiotic strains and especially strain combinations. Infection cases and detrimental effects are extremely rare and usually take place in subjects with a severe underlying condition, such as immunocompromised subjects or subjects with hepatitis (Besselink et al. 2008). For the general population there does not seem to be any risk of probiotic use, rather there may be benefits. Current probiotics have an excellent safety record, and many probiotic strains, such as those of the genera *Lactobacillus* and *Bifidobacterium*, are part of a normal healthy intestinal microbiota. These are not expected to pose any risk for the host’s health. The International Dairy Federation document on microbes with a long history of safe use in foods (Mogensen et al. 2002) and the Qualified Presumption of Safety (QPS) approach established by the European Food Safety Authority (EFSA 2007; see Section 31.3.2.2) support the safety of commonly used probiotics, including lactobacilli and bifidobacteria. Most current probiotics also have a long history of safe use and even with post-market monitoring data are available for many of them (Salminen et al. 2002). Given the importance of this issue, the safety of probiotics, in particular new probiotic strains and combinations that do not have a history of safe use and those belonging to species for which general assumption of safety cannot be made, requires a rigorous assessment. One challenge to be considered is the risk of transfer of resistance to antimicrobial agents to other microorganisms, and this is a real challenge for the present and future. Furthermore, the safety of probiotics in high-risk populations deserves particular attention.

**31.3.1 Safety Assessment of Probiotics in the EU**

In the EU the regulations on the safety and efficacy of probiotics largely differ depending on whether the probiotic is intended for humans or whether it is to be used as a feed additive. In the latter case the probiotic has to pass a detailed safety and efficacy assessment scheme. On the other hand, with human probiotics, the only cases requiring a mandatory safety assessment would be
either genetically modified strains (assessed according to the Directive 2001/18/EC [OJEU 2001] and Regulation (EC) No. 1829/2003 [OJEU 2003a]) or strains defined as novel foods (assessed according to Regulation (EC) No. 258/97 [OJEU 1997]). Since no notifications of either of these categories have thus far been submitted, there are no precedent cases of what could be regarded as a sufficient demonstration of safety in these cases.

31.3.2 Safety Assessment of Microbial Feed Additives

The relevant regulation is (EC) No. 1831/2003 (OJEU 2003b) on additives for use in animal nutrition. According to the regulation, each additive has to be assessed both for safety (taking into account the effects on the target animal, user, consumer, and environment) and efficacy. Detailed guidance documents on how to perform these assessments have been published by the EFSA Panel on Additives and Substances Used in Animal Feed (http://www.efsa.europa.eu/en/feedapguidance/feedaptechguidance.htm).

The target animal safety is assessed with the so-called tolerance test aiming to provide a limited evaluation of short-term toxicity of the additive and to establish a margin of safety. The duration of the studies depends on the target animal category (EFSA 2008a). Routinely, three groups of animals are used: a control group, the group receiving the additive at the maximum recommended dose, and a multifold-dose-level group. If possible at least a 10-fold overdose should be applied. The animals are followed on performance characteristics, hematology, and blood chemistry (not necessary, if at least a hundredfold overdose is applied), and for other parameters likely to be related to the biological properties of the additive.

The safety for the user (the person handling the feed) should be assessed by testing the formulated commercial product for respiratory toxicity (if the additive contains more than 1% of particles with a diameter ≤ 50 μm), skin and eye irritation, and skin sensitization.

The consumer safety concerns are related to the possibility that the microorganism used as a feed additive may produce during the fermentation unknown harmful metabolites, which could accumulate in animal products. The former Scientific Committee on Animal Nutrition formulated guidelines in 2001 evaluating microorganisms and enzymes for feed (European Commission 2001), and they are still applied. Currently, both genotoxicity tests (assays for point mutations and clastogenicity) and 90-day repeated dose-feeding studies on laboratory animals are formally required. These studies are not required if the product is intended for companion animals only.

The environmental safety of microbiological feed additives is evaluated on a case-by-case basis. Generally the intended use is not expected to affect the levels of the microorganisms in the receiving environment, and no specific studies are required.

31.3.2.1 The Specific Case of Antibiotic Resistance

In the EU the use of antibiotics as growth promoters is prohibited in order to control the emergence of antibiotic-resistant pathogenic strains harmful to human or animal health. However, even in the absence of antibiotics, a microorganism used as an additive can harbor transmissible resistance genes and could eventually transfer the resistance to pathogenic bacteria of either medical or veterinary importance. Accordingly specific data on the presence of transmissible antibiotic resistance genes in the microbial feed additives have been required since 2001, with a latest guidance document from EFSA dating from 2008 (EFSA 2008b). An additive must be screened against a panel of key antibiotics by determining the minimum inhibitory concentration (MIC). If the MICs exceed certain breakpoints, the cause of the resistance (mutation or an acquired resistance gene) has to be clarified.
31.3.2.2 Qualified Presumption of Safety

The discrepancy between the detailed safety requirements on microbiological feed additives and the virtual lack of any mandatory safety data of human food cultures was one of the reasons to introduce the concept of QPS as a tool for generic safety assessment of microbial strains notified to EFSA. Formally, the system was adopted by EFSA in 2007 (EFSA 2007). The QPS resembles to an extent the Generally Recognized As Safe (GRAS) system in the United States, but takes into account the specific European risk perceptions, such as concerns of transmissible antibiotic resistances.

The QPS approach means that a microorganism with an established safety record can be notified to EFSA without most of the safety studies formally required, provided that certain qualifications apply. Thus, for example, a LAB strain with a history of safe use can be notified as a feed additive without addressing target animal, consumer, and environmental safety aspects. The only qualification required would be the demonstrated lack of transmissible antibiotic resistance determinants.

Currently the QPS list includes, among others, bifidobacteria, lactobacilli, lactococci, pediococci, leuconostocs, oenococci, propionibacteria, certain bacilli, and yeasts that predominate in the EFSA notifications (Leuschner et al. 2010; EFSA 2010). This list is frequently updated, and further assessment is conducted.

31.4 Definition of Health Claims and Development of Legislation

There has been a long development of health claims resulting in greatly varying schemes in different countries worldwide. Health claims in functional foods were perhaps first defined in the regulation in Japan and came into force with the law on Foods for Specified Health Use (FOSHU). Thereafter, several countries have decided to regulate health messages or other messages on health benefits on foods or food components. In Europe the development has finally resulted in a common European-wide regulation on health claims in foods and this regulation concerns all EU member states. The main objectives of Regulation (EC) No. 1924/2006 of the European Parliament and of the Council of December 2006 on nutrition and health claims made on foods have been to ensure a high level of consumer protection, effective functioning of the internal market within the EU, fair competition within the food industry, and both stimulation and protection of innovations.

The EU strategy on health and nutrition has been based on precautionary politics. No foods have been banned or restricted because of their nutritional quality, but labeling information has been often required. Healthy European consumers are constantly encouraged to make healthy lifestyle choices, including choices of foods influencing nutrition. Labeling is a focus area for this strategy, and the health claims regulation has been designed to help enable individual consumers to make “informed choices.” The health claims legislation does not require the insertion of health claim information, but regulates the type of health or nutrition claims that may be made about food. Thus, the so-called functional foods have become synonymous with foods with health claims.

In the current EU regulation, the general objective is to harmonize rules on nutrition and health claims between EU member states. The regulation encompasses all aspects of health-related information in food products that are made in commercial communications, including labeling, presentations, advertising, and even brand names and trademarks. A health claim is any message related to these aspects of communication. The targets are to provide claims that 1) are not false or misleading, 2) do not challenge the nutritional adequacy of related foods and 3) do not encourage or condone excess consumption of foods, 4) do not challenge nutrition recommendations on a balanced diet, and 5) do not refer to bodily functions in an inappropriate way.
Health claims in Europe fall into three categories: function claims that are based on generally accepted scientific evidence, function claims that are based on newly developed scientific data, and reduction of disease risk claims or claims on growth and development of children.

The health benefits related to health claim regulation are meant to concern the common European healthy consumers or specific populations in Europe. A health claim is any message concerning health benefits or reduction in risk of disease. A probiotic health claim is any claim that states, suggests, or implies that a probiotic food has particular characteristics relating to its origin, nutritional properties, and impact on human health (WHO 2002).

31.5 Regulations on Probiotic Efficacy and Health Claims in Europe

Again, regarding animal probiotics, detailed regulatory framework on their efficacy is based on the Regulation (EC) No. 1831/2003. Regarding human health claims, Regulation (EC) No. 1924/2006 of the European Parliament and the Council on nutrition and health claims made on foods came into force on July 1, 2007. This was the first time that a regulation dealt with this issue, and the reason for implementing the regulation was that more and more products carried health claims. The intention is on the one hand to protect the consumer against misleading information, economical losses, or health problems and, on the other hand, to harmonize legislation in the member states. Meaningful health claims can help consumers make healthy food choices.

31.5.1 Specific Requirements of Regulation (EC) No. 1924/2006

The terms on health claims in the regulation, are defined in three different categories: first, the so-called Article 13 health claim, also referred to as “function claim,” which are claims referring to 1) the role of a nutrient or other substances in growth, development, and functions of the body; 2) psychological and/or behavioral functions; 3) slimming or weight control, or a reduction in the sense of hunger, or an increase in the sense of satiety, or the reduction of the available energy from the diet. The other two categories include the following areas for health claims: 1) reduction of disease risk claims; 2) claims referring to children’s development and health (Article 14 claim); 3) claims based on new and hitherto not established areas of health claims (including new innovative processes and products with diagnostic measures for the health claim). A reduction of disease risk claim is formed by “any health claim that states, suggests or implies that the consumption of a food category, a food or one of its constituents significantly reduces a risk factor in the development of a human disease” (EC 2006—Art. 2.2.6, Art. 14).

Additionally, the existing claims in different EU member states were collected by national authorities and submitted to the European Commission. The submitted claims are separately assessed to verify if scientific basis for such claims exists and in the positive case these are added to the commission decision and list of allowed European health claims.

For the existing Article 13 claims the member states had the possibility to submit national lists with claim proposals and scientific references to the EC until January 31, 2008, at the latest. The commission has asked EFSA, and specifically the Scientific Panel on Dietetic Products, Nutrition and Allergies, for the scientific assessment of individual health claims and also the submitted dossiers of other health claims. The claims and their conditions, as well as rejected claims with the reason for rejection, will be listed in a community register. This register shall be made available to the public (Article 20) (EC 2006).
Originally, a community register with all approved article 13.1 health claims was to be adopted by the Commission in 2010, but the claims assessment has been completed only in 2011. Therefore, the register will be published later. Until now, Commission decisions on individual applications have been published. The first commission decision on rejected health claims have appeared in October 2009, and these initiate the establishment of the community list of approved claims and rejected claims. The first list of authorized claims was published by the EU Commission in October 2009 (Commission Regulation (EC) No. 983/2009). These registers of allowed and rejected health claims are updated continuously.

31.6 Steps for Establishment of Probiotic Efficacy or Health Claims in the EU

31.6.1 Efficacy of Microbial Feed Additives

With zootechnical additives (intended to improve the growth performance), three studies on target animals showing a statistically significant positive effect on relevant parameters related to animal performance or on morbidity/mortality are usually required. Detailed instructions on the efficacy studies and their duration for each target animal category are given in the Technical Guidance on Tolerance and Efficacy Studies in Target Animals (EFSA 2008a).

31.6.2 Human Health Claims

The important areas to assess for health claims and probiotic health claims for each probiotics strain or strain combination are the following: characterization and efficacy especially in human intervention studies. These form the basis for probiotic assessment and the later establishment of potential human health claims (van Loveren et al. 2011). These steps are discussed in detail in the following sections.

In the framework of the EU Regulation on Nutritional and Health Claims made on Foods (Regulation (EC) No. 1924/2006) when assessing applications, EFSA has considered appropriate identification at the species and strain levels a restriction criteria for the further assessment of health claims related to probiotics. This points out at the need for proper species identification and characterization at the strain level (genetic typing), by using internationally accepted molecular methods. In addition, strains should be named according to the International Code of Nomenclature. In the context of this regulation, the purposes of characterization are to confirm the identity of the food/constituent that is the subject of the health claim, and to establish that the studies provided for substantiation of the health claim were performed with the food/constituent for which the claim is made. Characterization should also be sufficient to allow control authorities to verify that the food/constituent that bears a health claim in the market is the same that was the subject of community authorization (EFSA 2009b). According to the recommendations of the FAO (2006), and, although not mandatory, also recommended by EFSA, strains should also be deposited in an internationally recognized culture collection. These are important criteria that will assure the tracking and access of scientist and authorities to the strain and related information in case it is needed.

Therefore, proper identification of any investigated strain may constitute the critical starting point for probiotic studies. Nevertheless, a number of studies have reported that the identity of microorganisms isolated from probiotic products often does not correspond to the information stated on the product label (Gueimonde et al. 2004). A recent EU-funded project showed that 28% of the commercial probiotic cultures are misidentified already by their manufacturers or
distributors, which may explain the disagreements observed between the label information and the true identity of the isolated microorganisms. Accurate and reliable identification of probiotic strains is thus necessary to evaluate both the documented health benefits and the safety of probiotic products.

In the future, the increasing availability of genome sequences will allow genome-wide and/or multilocus phylogenetic analysis. During the last years the development of high-throughput sequencing technologies has enormously increased the sequencing capability, reducing significantly the sequencing costs. Although final genome assembly is still a very time-consuming task, the number of finished bacterial genomes and specially that of draft genomes is increasing very fast. In fact, some probiotic strain genomes have already been sequenced and in some cases the sequences have been deposited in public databases. In the future, the deposit of genomes from probiotic strains in commercial use, in public, or restricted access databases may overcome all the current limitations regarding identification at the species and strain levels. The genome sequence constitutes the best possible genetic fingerprint of a given strain. In addition, the availability of genomes of commercial strains would allow researchers and authorities a very rapid access to the information on potential traits of the strain in case new markers, related to efficacy or safety of probiotics, are identified in the future.

It is clear that strains used by the food industry and by scientists should be identified using molecular methods and up-to-date taxonomical nomenclature. In this respect, it is also important to make strains available in international culture collections. Even nowadays, many scientific articles are published without reporting data on the tested strains, hampering the progress of scientific development in this area and the assessment of the efficacy and safety of probiotics. The terminology of probiotics and health claims is presented in Figure 31.1, and terminology of health claims is presented in Figure 31.2.

31.6.3 Human Intervention Studies for Health Claims

To assist health claim applicants in preparing and submitting their applications for the authorization of health claims, EFSA and in particular its Scientific Panel on Dietetic Products, Nutrition and Allergies (NDA) have prepared a guidance document on gut health associated health claims (EFSA 2011a). The guidance document focuses on two key issues regarding the substantiation of health claims related to the gastrointestinal tract and immune system:

1) The claimed effects should be considered to be beneficial physiological effects.
2) The studies/outcome measures should be appropriate for the substantiation of health claims.

Health claim documentation is mainly based on human intervention studies conducted in the target population by using the food and ingredient in the intended dose level. This is especially true in case of claims intended for children’s health or claims associated with reduction in risk of disease. Before human intervention studies, it is important to establish the rationale for the probiotic strain or strain combination and to gain information on preclinical properties of the strain or strain combination.

For human intervention studies, it is most important to select the right target population that corresponds to the intended claim. It is as important to use the same dose of the food product or ingredient in the studies that is intended to be in the claim.

Human intervention studies can be classified in several ways, but the following hierarchy of study design forms a common basis:
Probiotics: Safety and Efficacy

Probiotic
“Live microorganisms, which when administered in adequate amounts, confer a health benefit on the host” (FAO 2002)

Health benefit
Condition that has been demonstrated to benefit the health of the host

Probiotic claim
Any representation that states, suggests or implies that a probiotic food has particular characteristics relating to its origin, nutritional properties, and health

Health claim
“Any claim that states, suggests or implies that a relationship exists between a food category, a food or one of its constituents and health” (EC 2006 – Art. 2.2.5)

Reduction of disease risk claim
“Any health claim that states, suggests or implies that the consumption of a food category, a food or one of its constituents significantly reduces a risk factor in the development of a human disease” (EC 2006 – Art. 2.2.6, Art. 14)

Health benefit
Condition that has been demonstrated to benefit the health of the host

Claim
“Any message or representation, which is not mandatory under Community or national legislation, including pictorial, graphic or symbolic representation, in any form, which states, suggests or implies that a food has particular characteristics” (EC 2006 – Art. 2.2.1)

Health claim
“Any claim that states, suggests or implies that a relationship exists between a food category, a food or one of its constituents and health” (EC 2006 – Art. 2.2.5)

Nutrition claim
“Any claim which states, suggests or implies that a food has particular beneficial nutritional properties due to the energy (caloric value) it provides; provides at a reduced or increased rate; or does not provide; and/or [due to] the nutrients or other substances it contains; contains in reduced or increased proportions; or does not contain” (EC 2006 – Art. 2.2.4)

Reduction of disease risk claim
“Any health claim that states, suggests or implies that the consumption of a food category, a food or one of its constituents significantly reduces a risk factor in the development of a human disease” (EC 2006 – Art. 2.2.6, Art. 14)

Claim referring to children’s development and health (EC 2006 – Art. 14.1)

Comparative nutrition claim
Nutrition claim that compares “the composition of the food in question with a range of foods of the same category, which do not have a composition which allows them to bear a claim, including foods of other brands” (EC 2006 – Art. 9.2)

Health claims other than those referring to the reduction of disease risk and to children’s development and health
“Health claims describing or referring to the role of a nutrient or other substance in growth, development and the functions of the body; psychological and behavioral functions; or […] slimming or weight-control or a reduction in the sense of hunger or an increase in the sense of satiety or to the reduction of the available energy from the diet” (EC 2006 – Art. 13.1)

Claim based on newly developed scientific evidence and/or that include protection of proprietary data (EC 2006 – Art. 13.5)
Probiotics: Safety and Efficacy

1) Human intervention studies, randomized controlled studies, other randomized studies (non-controlled), controlled (non-randomized) studies, other intervention studies
2) Human observational studies, cohort studies, case–control studies, cross-sectional studies, other observational studies, such as case reports
3) Other human studies dealing with the mechanisms of action

Human studies should be conducted according to international guidelines, and they should provide information on markers or factors that are important either as intermediate markers associated with clear end points in the disease or health area in the claimed effect. Such examples could be cholesterol levels in case of heart disease risk, or numbers of Streptococcus mutans bacteria in dental surfaces, oral pH, or dental plaque as risk factors for caries and tooth decay. Guidelines for gut and immune function as well as the information required for health claim applications are available (EFSA 2011a, 2011b).

31.6.4 Totality of Evidence for Health Claims in Foods

As specified in the European health claim regulation, all claims should be substantiated by taking into account the totality of the available scientific data and by weighing the evidence. This should be conducted considering the specific conditions of use. In particular, the total evidence should demonstrate the following matters: 1) The extent or importance of which the claimed effect of the food/constituent is relevant for human health. 2) Whether a scientific cause-and-effect relationship can be established between the consumption of the food or the food constituent and the claimed effect in humans (define, e.g., the strength, consistency, specificity, dose–response, and biological plausibility of the relationship). 3) The quantity of the food/ingredient and pattern of consumption required to obtain the claimed effect. The quantity and the daily portions should be such that they can be reasonably achieved as part of a recommended balanced diet in European countries. 4) The target population for which the claim is intended should be defined and the specific study populations in which the evidence was obtained should be representative of the target population (EFSA 2011a, 2011b).

31.7 Specific Challenges of Probiotics

31.7.1 Viability

The directions for the future of probiotics have some major challenges to overcome. The first very specific challenge for probiotics in the future is assessing viability. The current WHO definition of probiotics defines them as viable food supplements, but viability is defined by most regulatory authorities as culturability. Culturability itself depends on specific media and culture conditions. As demonstrated in human intestinal microbiota assessment studies, only a small part of the intestinal microbiota is culturable. However, it still is viable and will have an effect on human health.
Reliable determination of viability of bacteria in probiotic products is important for both human studies and product quality control. It is clear that the viability question of bacteria is not a simple question of cells being alive or dead. In most regulatory analysis, the plate count method has traditionally been used for controlling the viability of bacteria, but there are several disadvantages. First, the plate count method requires specific culture media and relatively long incubation times. The method is often hampered by technical difficulties, and the choice of enumeration medium and incubation conditions for specific species may be challenging. For many species residing in the human intestinal tract, a suitable growth medium is not even known. Furthermore, for fastidious microorganisms such as *Bifidobacterium* species of intestinal origin, which are increasingly used as probiotics, it may be difficult to find an optimal growth medium for reliable enumeration. Such bacteria have specific and unique nutritional and environmental requirements for optimal growth, and plate counts for certain strains may vary by several log units when grown on different nutrient-rich culture medium (Lahtinen et al. 2006a,b, 2008). It is not often feasible to attempt to find all potential growth media for a particular strain or purpose. The difficulties described above may lead to underestimation of the real probiotic counts. Another recently reported major challenge for the plate count method is the presence of the so-called dormant bacteria, which are unable to grow on conventional growth media but may nevertheless be measured as viable using cytological viability assays (Lahtinen et al. 2006b). Such dormant population may exist in many probiotic products and food starters, and similar population occurring in bile acid stressed bifidobacteria has been verified. Data from our laboratory suggest that probiotic bacteria in fermented product may become dormant during prolonged storage (Lahtinen et al. 2005, 2006b). We have therefore compared four different methods of enumeration. These included traditional plate counts, quantitative real-time PCR, fluorescent *in situ* hybridization, and commercial LIVE/DEAD (BacLight™) viability assay (L/D). Based on the comparison, we concluded that the choice of enumeration method for probiotic bacteria may have significant effects on the results of the analysis. The strain-specific properties and the objects of the analysis should be taken into account when future enumeration methods for different probiotic strains are chosen, and this should also be considered in regulatory control. One example exists in the animal feed area where the EFSA has approved an animal feed, where the probiotic supplement is only measurable as nonviable counts assessed by PCR. This may also provide potential for future products for which probiotic viability is defined by methods different from the commonly used culture methods.

### 31.7.2 Clinical Studies Demonstrating Efficacy of Probiotics in Healthy Subjects

Most probiotic studies in human subjects have been conducted in subjects who have been either ill or critically ill. Therefore, a further challenge is built in the form of an EU regulation in which the health claims are designed for normal healthy populations or populations at risk for specific disorders. An example is given by the scientific opinion on xylitol and tooth decay. Dental caries (tooth decay) is common in the normal healthy population in Europe. Dental caries and tooth decay are hallmarked by factors such as the extent of dental plaque, number of caries-causing organisms such as *Streptococcus mutans*, in dental surfaces. An additional factor is caused by acid production by such bacteria and the impact of acid on dental enamels. All these factors can be defined as risk factors in normal healthy populations. Using xylitol, a five-carbon polyol, in chewing gum has been demonstrated to influence the risk factors and also the end point dental caries, resulting in fewer missing or damaged dental surfaces after long-term chewing gum use (EFSA 2009a). Another example is formed by plant sterols and stanols, which have been demonstrated...
in normal populations to reduce elevated blood cholesterol levels. As there has been general con-
sensus that high cholesterol has been associated with coronary heart disease, the scientific opinion
of EFSA has allowed the claim that plant sterols and plant stanols help lower cholesterol, which is
associated with reduced risk of coronary heart disease. Similar areas should also be identified for
probiotic use and may become associated with health claims in the future.

31.7.3 Experiences of the Enforcement of Regulation (EC) No. 1924/2006

The regulation on health claims has been enforced in different European countries for 2 years,
but the practical measures are still developing and the EU Commission is still working on the
finalized format of health claims and a common European list of approved health claims. The
European Regulation (EC) No. 1924/2006 on claims made on foods will also have a great impact
on the European probiotic sector. The need for approval of any health claim made on foods, on
the basis of scientific evidence, promises to modify the marketing strategies used to communicate
the beneficial effects attributed to probiotic products within Europe. While the regulation on
probiotic health claims in other parts of the world may vary greatly, there is a clear trend for more
scientific substantiation of health claims, especially considering human studies.

On the basis of the European regulation, high-quality human intervention studies are needed
to substantiate a specific health claim for a certain product. This requirement for a number of
human studies focuses research efforts of both large multinational companies providing probiotic
strains and products as well as small- and medium-sized enterprises aiming at the production of
probiotic products with health claims.

31.8 Future Challenges in Regulatory Areas

The current health claim legislation varies significantly globally. Different safety and efficacy
requirements exist in the EU, Japan, Canada, Brazil, the United States, and other countries. In
Europe, the legislation is in the process of being harmonized throughout all EU member states.
However, differences still exist elsewhere and in spite of Codex work, other regulations are being
developed.

For safety assessment of probiotics, general guidelines have been issued by several countries.
In the EU the systematic assessment has resulted in a process called QPS. This assessment has
been conducted by EFSA, which also has published a list of microorganisms with safe history of
use. This list places the microorganisms in food into specific categories according to safety and
also allows the first screening for food grade bacteria for probiotic use. In the United States, the
GRAS notification exemplifies another system where the producer submits an expert dossier and
opinion on the safety of a particular product. The U.S. Food and Drug Administration (FDA)
then responds by a letter indicating either that it sees no concerns or that there may be challenges.
If no concerns are available, the probiotic will be placed on a public list with the letter from the
FDA describing the action taken (FDA 2011).

For health claims, the systems differ significantly across countries. In the EU the legislation is
now clear, while in the United States a more complex system exists. However, both systems require
proof of health benefits in human studies. In Japan, the FOSHU system also takes into account
smaller human studies complemented with studies in laboratory animals. In China, the system
becomes even more complicated as the provinces may have regulations that differ from those
established by the central government. Thus, the researcher or producer should be able to compare
the guidelines and to plan studies in human subjects in a manner that considers the specific points required by different countries or market areas.

Taken together, the health claim regulations require a lot of attention, and changes are still under way in several countries worldwide (see also Chapters 32 and 33). However, the basic principles appear to be similar and proof from well-designed human intervention studies will be more important in the future.

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Chapter 32

Probiotics Regulation in Asian Countries*

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* The chapter is a concerted effort of the Asian Federation of Societies for Lactic Acid Bacteria.
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<td>Guidelines on Probiotics</td>
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<td>Probiotic Claims</td>
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<td>Guidelines for the Evaluation of Probiotics for Drug Use (Bureau Circular No. 16 s. 2004 of BFAD, Philippines)</td>
</tr>
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<td></td>
<td>Guidelines for the Evaluation of Probiotic Food Use (Bureau Circular No. 16 s. 2004 of BFAD, Philippines)</td>
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<td>Regulating the Use of LAB as Probiotics</td>
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<td>Efficient Assessment of Probiotics to Health</td>
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<td>Appropriate Labeling</td>
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Acknowledgments
References
The guidelines for probiotic products in Asia are not harmonized; each country has its unique product regulation. Some are restrictive, whereas others are elaborative and specific.

### 32.1 China

Probiotics to most Chinese are not totally new, as they are found in traditional fermented vegetables, soy products, and dietary supplements. Probiotics in fermented dairy products was introduced only in the twenty-first century. The concept of health benefits of probiotics especially gut health was stressed when manufacturers promoted their yogurt (fermented milk) products. Probiotic yogurt has progressed from “concept” to “efficacy” by increasing the initial cell count and improving the storage condition to make sure that consumers can receive enough “alive healthy bacteria” to convey the health benefits it claims.

#### 32.1.1 Current Regulations and Standards Related to Probiotics

The production, trading, and distribution of food, food additives, food packaging materials, and other food-related products are strictly regulated in China by specific laws, regulations, and standards.

#### 32.1.2 Food Safety Law

The National People’s Congress issued the Food Safety Law in 2009, replacing the Food Sanitation Law which was issued by the Ministry of Health (MOH) in 1995. The Food Safety Law is the general and fundamental legislation in China that ensures food safety and protection of consumers.

The Food Safety Law states that the MOH is responsible for the overall coordination in food safety, which includes risk assessment, development of food safety standards, and publishing of food safety information. The authorities for quality supervision, administration of industry and commerce, and Food and Drug Administration (FDA) are responsible for supervision and management of the food production, food distribution, and the food safety in canteens and restaurants, respectively. The responsibilities of different authorities under the Food Safety Law are illustrated in Figure 32.1.

#### 32.1.3 Functional Food Regulation

The Functional Food regulation was initially developed by the MOH in 1996 and was revised in 2005 by the State Food and Drug Administration (SFDA). The definition of “Functional Food” was defined as a food that has special health functionalities. Functional Food is suitable for consumption by special groups of people with health benefits, but is not used for therapeutic purposes. The regulation provided the detailed requirements for registration of Functional Food.

#### 32.1.3.1 Approved List of Probiotics for Use in Functional Food

The term “probiotic” was legally presented in the Notice on the Evaluation of Functional Food with Fungus and Probiotics, issued by the MOH in 2001, Notice No. 84 (2001). The Notice was developed based on the Food Sanitation Law and the Regulation on Functional Food. It regulates
the detailed documentation requirements, production, authorized testing centers, and the list of fungus and probiotics for use in Functional Food. As shown in Table 32.1, the original list has four species of yeast and seven species of mold, but only nine species are probiotics. New probiotic species can be added into the list after evaluation and approval by the MOH. One species, *Lactobacillus reuteri*, has been added to the list so far.

### Table 32.1  Approved List of Probiotics for Use in Functional Food

<table>
<thead>
<tr>
<th>List of Fungus for Use in Functional Food</th>
<th>List of Probiotics for Use in Functional Food</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saccharomyces cerevisiae</td>
<td>Bifidobacterium bifidum</td>
</tr>
<tr>
<td>Candida atilis</td>
<td>Bifidobacterium infantis</td>
</tr>
<tr>
<td>Kluyveromyces lactis</td>
<td>Bifidobacterium longum</td>
</tr>
<tr>
<td>Saccharomyces carlsbergensis</td>
<td>Bifidobacterium breve</td>
</tr>
<tr>
<td>Paecilomyces hepial Chen et Dai, sp. nov</td>
<td>Bifidobacterium adolescentis</td>
</tr>
<tr>
<td>Hirsutella hepial Chen et Shen</td>
<td>Lactobacillus bulgaricus</td>
</tr>
<tr>
<td>Ganoderma lucidum</td>
<td>Lactobacillus acidophilus</td>
</tr>
<tr>
<td>Ganoderma sinensis</td>
<td>Lactobacillus casei subsp. casei</td>
</tr>
<tr>
<td>Ganoderma tsugae</td>
<td>Streptococcus thermophilus</td>
</tr>
<tr>
<td>Monascus anka</td>
<td></td>
</tr>
<tr>
<td>Monascus purpureus</td>
<td></td>
</tr>
</tbody>
</table>
Since 2003, SFDA has taken over the responsibilities of the management of Functional Food from the MOH. The regulation on Functional Food has been revised and issued in 2005. After this change, the two lists remain valid, but it is clear that the new regulation no longer approves addition of new probiotic species to the list. Approval on the use of new probiotic species will be evaluated case-by-case, together with the end products.

### 32.1.3.2 Health Claims of Functional Food with Probiotics

SFDA has published a list of permitted health claims as shown in Table 32.2. Most of the probiotic Functional Food products have been approved to have the health claims “enhance immunity” and/or “regulate gastrointestinal tract flora” among the 27 permitted health claims.

### 32.1.4 Novel Food Regulation

The first regulation on Novel Food was issued by the MOH in 1990. According to the regulation, the scope of Novel Food includes both food ingredients and end food products that are newly developed, newly discovered, or newly introduced, which are not part of the traditional diet or

<table>
<thead>
<tr>
<th>Claims</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Enhance immunity</td>
<td>13. Increase bone density</td>
</tr>
<tr>
<td>3. Assist in memory improvement</td>
<td>15. Assist in protecting against chemical injury to the liver</td>
</tr>
<tr>
<td>5. Facilitate lead excretion</td>
<td>17. Eliminate skin chloasma</td>
</tr>
<tr>
<td>6. Moisten and clean throat</td>
<td>18. Improve skin water content</td>
</tr>
<tr>
<td>7. Improve sleep</td>
<td>19. Improve skin oil content</td>
</tr>
<tr>
<td>9. Alleviate physical fatigue</td>
<td>21. Facilitate digestion</td>
</tr>
<tr>
<td>10. Enhance anoxia endurance</td>
<td>22. Facilitate feces excretion</td>
</tr>
<tr>
<td>11. Assist in irradiation hazard protection</td>
<td>23. Assist in protecting against gastric mucosa damage</td>
</tr>
<tr>
<td>12. Improve child growth and development</td>
<td></td>
</tr>
<tr>
<td>24. Weight loss</td>
<td>26. Assist in blood sugar reduction</td>
</tr>
<tr>
<td>25. Assist in blood lipids reduction</td>
<td>27. Assist in blood pressure reduction</td>
</tr>
</tbody>
</table>
### Table 32.3  Probiotic Strains with Novel Food Approval

<table>
<thead>
<tr>
<th>Order</th>
<th>Species</th>
<th>Strain</th>
<th>Manufacturer</th>
<th>Date of Approval</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>Lactobacillus rhamnosus</em></td>
<td>LGG/ATCC 53103</td>
<td>Valio</td>
<td>2007-1-11</td>
<td>food in general</td>
</tr>
<tr>
<td>2</td>
<td><em>Lactobacillus rhamnosus</em></td>
<td>HOWARU Rhamnosus/ HN001</td>
<td>Danisco</td>
<td>2007-8-6</td>
<td>food in general</td>
</tr>
<tr>
<td>3</td>
<td><em>Bifidobacterium animalis</em></td>
<td>Bb12</td>
<td>Chr. Hansen</td>
<td>2007-2-13</td>
<td>food in general</td>
</tr>
<tr>
<td>4</td>
<td><em>Bifidobacterium lactis</em></td>
<td>HOWARU Bifido/HN019</td>
<td>Danisco</td>
<td>2007-8-6</td>
<td>food in general</td>
</tr>
<tr>
<td>5</td>
<td><em>Bifidobacterium lactis</em></td>
<td>Bi-07/SD5220</td>
<td>Danisco</td>
<td>2007-8-6</td>
<td>food in general</td>
</tr>
<tr>
<td>6</td>
<td><em>Lactobacillus acidophilus</em></td>
<td>NCFM/SD5221</td>
<td>Danisco</td>
<td>2006-11-21</td>
<td>food in general</td>
</tr>
<tr>
<td>7</td>
<td><em>Bifidobacterium animalis</em></td>
<td>BE80</td>
<td>Danone</td>
<td>2007-2-13</td>
<td>food in general</td>
</tr>
<tr>
<td>8</td>
<td><em>Lactobacillus acidophilus</em></td>
<td>DSM13241</td>
<td>–</td>
<td>2008-5-26</td>
<td>dairy, Functional Food</td>
</tr>
<tr>
<td>9</td>
<td><em>Lactobacillus paracasei</em></td>
<td>GM080</td>
<td>–</td>
<td>2008-9-9</td>
<td>dairy, Functional Food, beverage, biscuit, confectionery, ice cream</td>
</tr>
<tr>
<td>10</td>
<td><em>Lactobacillus acidophilus</em></td>
<td>R0052</td>
<td>–</td>
<td>2008-9-9</td>
<td>Functional Food</td>
</tr>
<tr>
<td>11</td>
<td><em>Lactobacillus rhamnosus</em></td>
<td>R0011</td>
<td>–</td>
<td>2008-9-9</td>
<td>Functional Food</td>
</tr>
<tr>
<td>12</td>
<td><em>Lactobacillus plantarum</em></td>
<td>299v</td>
<td>–</td>
<td>2008-9-9</td>
<td>dairy, Functional Food</td>
</tr>
<tr>
<td>13</td>
<td><em>Lactobacillus plantarum</em></td>
<td>CGMCC No. 1258</td>
<td>–</td>
<td>2008-9-9</td>
<td>beverage, ice cream, Functional Food</td>
</tr>
<tr>
<td>14</td>
<td><em>Lactobacillus plantarum</em></td>
<td>ST- III</td>
<td>–</td>
<td>2009-9-27</td>
<td>dairy, LAB drinks</td>
</tr>
</tbody>
</table>
only consumed in remote territories of China. The objective is to evaluate the safety of the food, but not for health function and related claim. From 2006 to the end of 2007, seven probiotic strains have been approved as Novel Food. After Dec 1, 2007, a new Novel Food regulation came into force. The major changes are: (1) the new regulation approves food ingredients only, not for end food products anymore; (2) the new approvals and the general specifications are published by the MOH official announcements, whereas the previous regulation only issued certificates to the companies. In the following 2 years, another seven probiotic strains have been approved under the new regulation. The 14 probiotics with Novel Food approved are summarized in Table 32.3.

### 32.1.5 Approved List of Culture Species for Use in General Foodstuff

Chinese MOH issued “The list of culture species for use in foodstuff” in April 2010. The list has 21 culture species, including six *Bifidobacterium*, 14 *Lactobacillus*, and one *Streptococcus*.

Please be aware that there are two supplementary comments (Notes attached to Table 32.4).

Note 1 states that “Cultures traditionally used in food processing are still permitted for use; novel strain is regulated under Novel Food regulation.” This means that traditional starter cultures

#### Table 32.4 List of Approved Culture Species for Use in Foodstuff

<table>
<thead>
<tr>
<th>Bifidobacterium</th>
<th>Lactobacillus</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bifidobacterium adolescentis</em></td>
<td><em>Lactobacillus acidophilus</em></td>
</tr>
<tr>
<td><em>Bifidobacterium animalis (Bifidobacterium lactis)</em></td>
<td><em>Lactobacillus casei</em></td>
</tr>
<tr>
<td><em>Bifidobacterium bifidum</em></td>
<td><em>Lactobacillus crispatus</em></td>
</tr>
<tr>
<td><em>Bifidobacterium breve</em></td>
<td><em>Lactobacillus delbrueckii subsp. bulgaricus (Lactobacillus bulgaricus)</em></td>
</tr>
<tr>
<td><em>Bifidobacterium infantis</em></td>
<td><em>Lactobacillus delbrueckii subsp. lactis</em></td>
</tr>
<tr>
<td><em>Bifidobacterium longum</em></td>
<td><em>Lactobacillus fermentium</em></td>
</tr>
<tr>
<td></td>
<td><em>Lactobacillus gasseri</em></td>
</tr>
<tr>
<td><em>Streptococcus</em></td>
<td><em>Lactobacillus helveticus</em></td>
</tr>
<tr>
<td><em>Streptococcus thermophilus</em></td>
<td><em>Lactobacillus johnsonii</em></td>
</tr>
<tr>
<td></td>
<td><em>Lactobacillus paracasei</em></td>
</tr>
<tr>
<td></td>
<td><em>Lactobacillus plantarum</em></td>
</tr>
<tr>
<td></td>
<td><em>Lactobacillus reuteri</em></td>
</tr>
<tr>
<td></td>
<td><em>Lactobacillus rhamnosus</em></td>
</tr>
<tr>
<td></td>
<td><em>Lactobacillus salivarius</em></td>
</tr>
</tbody>
</table>

**Note 1:** Cultures traditionally used in food processing are still permitted for use; novel strain is regulated under Novel Food regulation.

**Note 2:** The cultures used in infant and young children’s (0–3 years old) formula are regulated under the current rule; the list will be established separately.
used for production of fermented food are not affected. However, Chinese food industries and supervision authorities tend to rely on written regulation. This statement is difficult to interpret and implement and it seems that the list is still too short and needs to be further extended.

Note 2 states that “The cultures used in infant and young children’s (0–3 years old) formula are regulated under the current rule; the list will be established separately.” This indicates that: (1) the list is not applicable to 0–3 years baby formula; (2) the strains with Novel Food approval and without restriction for use in 0–3 years baby formula can still be used; and (3) the MOH will establish a separate list for 0–3 years baby formula later. In fact, the MOH opened a draft list in March 2009 for public comments. The final list will be developed based on the draft. The message is that the list for 0–3 years baby formula will be evaluated at strain level.

32.1.6 Food Safety Standards

According to the new Food Safety Law, food safety standards are mandatory and exclusive. Besides the food safety standards, no other mandatory standards for food shall be developed. After the melamine crisis in 2009, the revision and development of dairy safety standards became urgent and a prioritized task. The MOH established a working group to consolidate and complete the existing dairy standards. In April 2010, the MOH announced 66 dairy standards, including 14 product standards, 2 Good Manufacturing Practices (GMP) standards, and 50 analytical standards. They became the first group of food safety standards in China. Key provisions related to probiotics in dairy standards are as presented in the following sections.

32.1.6.1 Product Standard GB19302-2010 “Fermented Milk”

The standard was revised based on the previous standard GB19302-2003 “Hygienic Standard for Yogurt” and the previous product standard GB2746-1999 “Yogurt.” It also referred to Codex Standard for Fermented Milks. Thus, the scope of the new standard extended from “Yogurt” to “Fermented Milk.” For starter cultures, the standard specified the requirements as “Lactobacillus bulgaricus (Lactobacillus delbrueckii subsp. bulgaricus), Streptococcus thermophilus and other cultures permitted by MOH.” This statement allows the industry to use the culture presented in the “List of culture species for use in foodstuff.” However, because the list does not include yeasts and many lactic acid bacteria (LAB) species, some typical fermented milk products, such as Kefir and Kummy, are difficult to develop unless the species can be considered as traditional culture following Note 1 of the list.

The requirement for the number of LAB in non-heat-treated fermented milk should be \( \geq 1 \times 10^6 \) CFU per gram per milliliter in the standard.

32.1.6.2 Product Standard GB10765 “Infant Formula” and GB10767 “Older Infant and Young Children Formula”

The term “probiotics” is defined in the two standards. The standards state “total plate count is not applicable to products with live culture” (aerobic and facultative anaerobic probiotics). The live probiotics in the product should be \( \geq 1 \times 10^6 \) CFU per gram per milliliter.

32.1.6.3 Standard of Analysis of LAB in Food

The standard GB4789.35 “Food microbiological examination: Lactic acid bacteria” was published together with other dairy standards in 2010. This is the official method used to identify and
count the number of live LAB in food products. Three typical media—MRS, modified MRS with Li–Mupirocin, and MC—are used to count total number of LAB, *Bifidobacterium*, and *S. thermophilus*, respectively.

### 32.2 India

There is no regulation as yet in India for dietary supplements. Probiotics are available as omega fortified food, vitamin and mineral enriched cereals, fortified salt and wheat flour, ginseng fortified health tea, chocolate bars, and plant sterol fortified functional cooking oils. As drug or biological product, a probiotic would be treated as a new chemical entity under the Schedule Y requirement of Drugs and Cosmetics Act, 1940; violation of any such Act may be punishable as per the description under the Act. Approval for such product would be from the Drugs Controller General of India (DCGI).

Therefore, if a probiotic is marketed as such, only the regulations of the country of import would apply as of now. If the product is a biotech one, it will have to be approved by the highest committee, Genetic Engineering Approval Committee (GEAC), under the Ministry of Environment and Forests. Mashelkar Committee, an inter-ministerial committee under the Ministry of Environment and Forests, made recommendations for streamlining and reducing the process and time taken for clearance of recombinant pharma products. According to these recommendations for importing and marketing in India, any finished biotech product or bulk for making finished formulations would be passed on to DCGI by GEAC for further approvals and monitoring. However, for import and marketing of products derived from the living micro-organisms as drugs and brought in bulk and/or finished formulations, GEAC will have no role to play and it will be the sole responsibility of DCGI to deal with this area. If the product is to be marketed for domestic use as dietary supplement, there is no regulation. For export to foreign countries, the guidelines/regulations of those countries for dietary supplements will have to be followed. Approval/restriction of use of probiotics in food may be decided by the concerned food regulatory authorities.

### 32.2.1 Indian Council of Medical Research Guidelines for Probiotics

The Indian Council of Medical Research (ICMR) guidelines deal with the use of probiotics in food and provide requirements for assessment of safety, efficacy, health claims, and labeling of such products intended for use in humans. Thus, issues required to be considered according to the guidelines are as follows.

#### 32.2.1.1 Genus, Species, and Strain Identification

#### 32.2.1.1.1 In Vitro Tests to Screen Potential Probiotic Strains

The following battery of *in vitro* tests with standard methodology is recommended for screening potential probiotic strains:

1. Resistance to gastric acidity
2. Bile acid resistance
3. Adherence to mucus and/or human epithelial cells and cell lines
4. Antimicrobial activity against potentially pathogenic bacteria
5. Ability to reduce pathogen adhesion to surfaces
6. Bile salt hydrolase activity

32.2.1.2 Evaluation of Safety of Probiotics for Human Use

In recognition of the importance of assuring safety, even among groups of bacteria that are Generally Recognized as Safe (GRAS), probiotic strains are required to be characterized at a minimum with the following tests:

1. Determination of antibiotic resistance patterns.
2. Assessment of certain metabolic activities (e.g., d-lactase production, bile-salt deconjugation).
3. Assessment of side-effects during human studies.
4. Post-market surveillance of adverse incidents in consumers.
5. If the strain under evaluation belongs to a species that is a known mammalian toxin producer or of hemolytic potential, it must be tested for toxin production and hemolytic activity, respectively. Assessment of lack of infectivity by a probiotic strain in immunocompromised animals would be an added measure.

32.2.1.3 In Vivo Studies in Animals for Safety

Assessment of the acute, subacute, and chronic toxicity of ingestion of extremely large amounts of probiotics should be carried out for all new strains.

32.2.1.4 In Vivo Studies in Animal Models and Humans

To substantiate in vitro effects, appropriate, validated animal models may be utilized first, before human trials. The principal outcome of efficacy studies on probiotics should be proven with similar benefits in human trials, such as statistically and biologically significant improvement in condition, symptoms, signs, well being or quality of life, reduced risk of disease or longer time to next occurrence, or faster recovery from illness. Each should have proven correlation with the probiotics tested. Standard methods for clinical evaluations comprise of Phase 1 (safety), Phase 2 (efficacy), Phase 3 (effectiveness), and Phase 4 (surveillance). If the probiotic food has a record of documented long and safe use outside the country, the data regarding this may be reviewed and deemed as sufficient to allow its marketing within the country. However, labeling of health benefits may be required to be evaluated in a different manner. While taking into account studies done abroad, efficacy studies of probiotics should also be conducted on the Indian population. It is recommended that such ‘bridging’ of human trials be repeated at more than one Center for verification of health claim(s). Adverse effects, if any, should be monitored and incidents reported to the appropriate authority.

32.2.1.5 Labeling Requirements

In addition to the general labeling requirements under the food laws, the following information should also be mentioned on the label:

a. Genus, species, and strain designation following the standard international nomenclature.
b. The minimum viable numbers of each probiotic strain should be specified at the level at which efficacy is claimed and at the end of shelf-life.
Probiotics Regulation in Asian Countries

32.2.2 Principles of Ethical Conduct

In research ethics including research on probiotics, three basic ethical principles have been identified—respect for persons, beneficence, and justice. The research proposal/project/trial should be reviewed and approved by a properly constituted Ethics Committee. The consent of the participants must be obtained before research is undertaken except in exceptional circumstances described in the National Statement.

32.2.2.1 Issues Related to Research Participants

With respect to clinical trials of the product, the ethical principles which apply for the protection of the trial participant should be observed. The main objective of this is to minimize harm to the participant. To justify this, a thorough literature survey should be done to note the possible side-effects to weigh the risk–benefit ratio appropriately as many ethical guidelines consider that harm to an individual should not be done at the expense of society’s benefit.

32.2.2.2 Role of Ethics Committees

The ethics committees comprise medical, non-medical scientists, and non-scientists. The scientists in the committee have an obligation to study the scientific contents of the proposal carefully. The committee would approve a project only after review of the side-effects that are highlighted in the proposal, based on which it would determine whether the harm to the participant is minimal. Like other trials, emphasis has to be given on essentiality, risk–benefit ratio, selection of participants, conduct of the study, privacy and confidentiality, compensation, and protection of research participants, which are equally important for review by the institutional ethics committees to safeguard the interests of these participants. If a subject expert needs to be consulted, then such a person could be co-opted to give comments. In multicentric trials, approval needs to be taken from the independent ethics committee (IEC) of each trial site as it would not only take care of the details of the proposal in the context of the local culture, but will also be responsible for the conduct of the trial for which it has given the approval. The IEC would have to review the proposal in the light of the revised ‘Ethical Guidelines for Biomedical Research on Human Participants’ brought out by the ICMR in 2006.

These guidelines are intended for investigators, health policy-makers, members of ethical review committees, and others who have to deal with ethical issues that arise in epidemiology. They may also assist in the establishment of standards for ethical review of epidemiological studies.

32.3 Indonesia

Probiotic product was first introduced in Indonesia in 1991 when Yakult was launched. With the factory (PT Yakult Indonesia Persada) located at Cicurug, Sukabumi and the capacity of 1,800,000 bottles per day, this fermented milk drink using Lactobacillus casei Shirota strain dominates the probiotic market in Indonesia until now. The next probiotic product is VitaCharm Multi
Probiotic ABC, launched in 2007 by PT Ultra Prima Artaboda using three probiotic strains for gut health, that is, Acidophilus Digestiva®, Casei Immunita®, and also Bifido Defensia®. Probiotic yogurt with Activia brand of DANONE is the first probiotic yogurt in Indonesia. This yogurt contains live bacteria Probiotic Exclusive ActiRegularis™.

The other probiotic product is milk formula Dancow produced by Nestlé, which uses the strain Lactobacillus PROTECTUS®. This product is also supplemented with fructo-oligosaccharide and inulin as prebiotic.

In the market, there are also powder probiotic as supplement in capsule form, such as Rillus and SynBio, as well as chewing gum (BioGaia) sold by PT Kalbe Farma.

Like many other countries in Asia, marketing of functional foods (including probiotic foods) with many kinds of health claims in Indonesia is increasing year by year. This situation prompted the government to establish a regulation with the main purpose of controlling distribution of functional foods and to protect consumers from excessive information in the label and advertising related to health claims. In January, 2005, the National Agency for Food and Drug Control (NAFDC) Republic of Indonesia established a regulation to control functional food (BPOM, 2005). According to this regulation, functional food is defined as processed food containing one or more ingredients which, based on scientific evidence, have certain physiological function, health benefit, and are safe for consumption. According to this regulation (article 5), ingredients of functional food can be categorized as follows: (1) vitamin, (2) mineral, (3) alcoholic sugar, (4) unsaturated fatty acid, (5) peptide and special protein, (6) amino acid, (7) food fiber, (8) prebiotic, (9) probiotic, (10) choline, lecithin, and inositol, (11) carnitine and squalene, (12) isoflavones (soy bean), (13) phytosterol and phytostanol, (14) polyphenol (tea), and (15) another functional ingredient which will be defined later. Manufacturers producing functional foods should implement GMP and Hazard Analysis and Critical Control Points systems (article 6).

Related to the claims, there are three kinds of claim that can be obtained for functional foods, and these claims can be included on label and advertisement (according to the existing regulation on label and advertisement). These claims are ingredient (nutrient) claim, functional claim, and health claim (such as reduction risk of diseases) which should be approved by NAFDC Republic of Indonesia, based on scientific and clinical evidences.

Claims than can be obtained for probiotic products are ingredient claim (with a dose of 10^6–10^8/day) and functional claim, that is, to maintain the health of gastrointestinal tract. Furthermore, strains that can be used as probiotics are species of Lactobacillus (except L. bulgaricus) and Bifidobacterium. Probiotic food label and advertisement must include this explanation.

1. Description of genus, species, and strain of probiotic used
2. Minimum number of viable cells at the end of shelf-life
3. The suitable size to describe the probiotic dose which is related to the claims
4. Information about the accurate number of probiotic related to the physiological effect
5. Suitable storage condition
6. Detailed information of the company for consumer to contact with

32.4 Japan

32.4.1 Food for Specified Health Uses (FOSHU) Definition

Food for Specified Health Uses (FOSHU) was set up by the MOH, Labor and Welfare (MHLW) in 1991 as a regulatory system to review and approve label statements regarding effects of foods on
the human body. FOSHU is defined as (http://www.mhlw.go.jp/topics/bukyoku/iyaku/syoku- anzen/hokenkinou/hyouziseido-1.html)

1. Foods with active constituents that affect the physiological function and biological activities of the body
2. Foods used as daily diet and claimed to promote a specified health benefit and can obtain such a benefit
3. Food products that are evaluated individually according to their substantiation, validity, safety, and quality, and finally approved by the government

32.4.2 FOSHU Criteria
As a FOSHU product, the following requirements are to be met (http://www.nihs.go.jp/hse/food-info/mhlw/news/2005/050203/050203-8.pdf):

1. Must be foods that the consumer hopes will improve one's dietary habits and will help to maintain and promote health.
2. The specified health use of the food or the functional component must be backed up by scientific evidence (through medical and nutritional studies).
3. Adequate amount of intake must be indicated according to medical and nutritional studies.
4. The safety of the food and the functional ingredient must be clearly understood from the material attached to the application.
5. The functional ingredient must be defined in physical, chemical, and physiological properties and must have its own testing method, including quantitative and qualitative analyses.
6. The nutritional component of FOSHU should not be fundamentally different from a similar food that is non-FOSHU.
7. Must be food that is eaten on a daily basis, not something that is rarely eaten.
8. The functional ingredient must not be included in the list of Primary Medical Ingredients of the ministry’s notice from 1971.

32.4.3 FOSHU Application
Those who wish to apply for FOSHU labeling on their products are required to submit the following documentation to the MHLW as indicated below (http://www.nihs.go.jp/hse/food-info/mhlw/news/2005/050203/050203-11.pdf):

1. Sample of the entire package with labels and health claims
2. Documentation that demonstrates clinical and nutritional proof of the product and/or functional component for the maintenance of health
3. Documentation that demonstrates clinical and nutritional proof of the amount of the product and/or functional component ingested
4. Documentation concerning the safety of the product and functional component, including additional human studies about the eating experience
5. Documentation of the physical and biological characteristics of the product and functional component
6. Methods of qualitative and quantitative analytical determination of its functional component, and analytical assay results of the component in the product
7. Report on the analysis of the designated nutrient constituents and energy content of the product
8. Statement of production methods, list of factory equipment, and an explanation of the quality control system

Mandated FOSHU documentation can be summarized into three essential requirements for FOSHU approval: (1) effectiveness based on scientific evidence, including clinical studies; (2) safety as assessed from historical consumption pattern data and additional safety studies conducted in humans; and (3) analytical determination of the functional component responsible for the beneficial physiological effect.

Regarding effectiveness, documents should be prepared based on substantiation not only by human intervention studies but also by in vitro metabolic and biochemical studies and animal studies. These data should demonstrate statistically significant differences. Basically, a human study should be conducted by using the food in question over a reasonably long-term period (e.g., more than 2 or 3 months). A human study should be approved by a committee on ethics in consideration of the protection of human rights, in accordance with the principles of the Helsinki Declaration. The study should also be well designed, for example, using an appropriate functional marker, appropriate sample size, and a sufficient number of subjects to prove statistically significant differences. All available literature regarding the related functional components, the related foods, and the related functions should be reviewed.

Any new scientific evidence used to support health-related claims must be published by a suitably qualified journal with expert referees who can review the evidence. Generally, more than two human studies are required for different targeted individuals.

As for safety, both in vivo and in vitro studies should be carried out to obtain preliminary data confirming the food’s safe ingestion by human consumers. Even if an effective component has been consumed as food by a reasonable number of individuals during certain periods, safety data for human consumers should be required for at least three times the minimum effective dosage. The literature regarding related functional components should be reviewed. If the related literature suggests an especially undesirable or adverse health effect, the report should be included as a reference with the scientific explanation or the human study that confirms the product’s safety for human consumers.

Concerning analysis, documentation of the methods of analysis of related functional components should submit the suitable and reliable methods of quantitative and qualitative analytical determination. As additional documentation, the stability of related functional components should be confirmed. The effective components and other components with an undesirable or adverse health effect should be confirmed to demonstrate the specified amount through the use of suitable analytical methods. If a product is in the form of tablets or capsules, experiments should be conducted regarding its characteristics of disintegration or dissolution.

It is important within the evaluation process that both the benefits and safety of a given functional food differ from those of a medicine. Functional foods are designed to target healthy people or people in the preliminary stage of a disease or a borderline condition of at-risk groups. Therefore, effectiveness for these people may be reduced as compared with medicine for patients. Generally, foods with functionality have been historically consumed by people and thus, can be regarded as safer than innovative medicine.
32.4.4 FOSHU Labeling

As final products, the following items must be labeled on a FOSHU package after approval (http://www.nihs.go.jp/hse/food-info/mhlw/news/2005/050203/050203-8.pdf):

1. Foods with Health Claims (FOSHU)
2. Approved function claims
3. Nutrition facts
4. List of ingredients
5. Total content
6. Standard daily administration
7. Methods of ingestion and warning concerning those methods
8. Percentage of the active substance included in the daily amount of administration to the substance’s set DRI
9. Warning concerning cooking methods or preservation
10. FOSHU logo of approval
11. Health claims involving FOSHU must not express medical claims such as “prevent,” “cure,” “treat,” and “diagnose.” Here are some examples of permitted and prohibited claims for human diseases: (1) Maintain or improve a marker determined by self-diagnosis or a health checkup. An example of a permitted claim is as follows: “This product helps to maintain normal blood pressure, blood sugar, or cholesterol.” An example of a prohibited claim is as follows: “This product improves hypertension.” (2) Maintain physiological function and organ function of the human body in good condition or improve them. An example of a permitted claim: “This product enhances the absorption of calcium” or “This product helps to improve the movement of the bowel.” An example of a prohibited claim: “This product is an effective food for intoxication” or “Enhance fat metabolism.”

32.4.5 FOSHU Health Claims Classification

The existing health claims on FOSHU can be classified into eight groups according to the health claims, such as gastrointestinal conditions, mineral absorption, blood pressure, or blood cholesterol.

The health benefits of probiotics maybe classified into immunoregulatory activity and effect to regulate the functions of the intestines. For the former benefit, probiotic products are not yet recognized as a FOSHU because the endpoints are not clear. Their health benefits in reducing disease development risks are therefore limited to calcium intake and osteoporosis and folic acid neural tube defect (http://www.mhlw.go.jp/topics/bukyoku/iyaku/syoku-anzen/hokenkinou/hyouziseido-1.html#sippei). For the regulation of intestinal functions, the benefits of alleviating the symptoms of inflammatory bowel disease, severe constipation requiring medical attention, and diarrhea are excluded from consideration, and only limited appeals for improvements in the tendency to cause constipation are allowed. On the other hand, appeals related to improvements in the intestinal environment, for example, an increase in bifidobacteria counts, a decrease in harmful bacteria, or an increase in metabolites seem to be allowed with more flexibility.

Table 32.5 lists the names of probiotic strains contained in foods approved as FOSHU and their health benefits (http://www.mhlw.go.jp/topics/bukyoku/iyaku/syoku-anzen/hokenkinou/xls/hyouziseido-1a.xls). As can be seen, each product has different claims. The claims should be
Table 32.5  Approved Health Claims Related to Probiotic Products

<table>
<thead>
<tr>
<th>Strain Name</th>
<th>Approved Health Claim</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Lactobacillus GG</em></td>
<td>This drink is produced by using fermented yogurt with <em>Lactobacillus</em> GG, which reaches one’s intestines in an active state so as to help increase intestinal bifidobacteria and lactobacilli. This promotes the maintenance of a good intestinal environment and regulates the gastrointestinal condition.</td>
</tr>
<tr>
<td><em>Lactobacillus GG</em></td>
<td>With the help of <em>Lactobacillus</em> GG, which arrives in our intestines alive, the number of good bacteria increases and the number of bad ones decreases. This improves the gastrointestinal environment and keeps the intestines healthy.</td>
</tr>
<tr>
<td><em>Bifidobacterium longum</em> BB536</td>
<td>This yogurt contains living bifidobacteria (<em>Bifidobacterium longum</em> BB536) and helps increase intestinal bifidobacteria. It helps maintain a good intestinal environment and regulate the gastrointestinal condition.</td>
</tr>
<tr>
<td><em>Bifidobacterium longum</em> BB536</td>
<td>Because this product includes live bifidobacteria, it helps increase the bacteria in the intestines and maintains a healthy gastrointestinal environment.</td>
</tr>
<tr>
<td><em>Lactobacillus delbrueckii</em> subsp. bulgaricus 2038 and <em>Streptococcus salivarius</em> subsp. thermophilus 1131</td>
<td>The lactobacillus LB81 in this yogurt helps regulate the balance of intestinal microflora to maintain a good gastrointestinal condition.</td>
</tr>
<tr>
<td><em>Lactobacillus acidophilus</em> SBT-2062 and <em>Bifidobacterium longum</em> SBT-2928</td>
<td>The acidophilus and <em>Bifidobacterium</em> in this yogurt help improve the intestinal environment and regulate the gastrointestinal condition.</td>
</tr>
<tr>
<td><em>Lactobacillus casei</em> Shirota</td>
<td><em>L. casei</em> Shirota reaches our intestines in an active state. This increases the number of good bacteria and decreases bad ones in the intestines and helps regulate the balance of intestinal microflora that lead to the maintenance of a good gastrointestinal condition.</td>
</tr>
<tr>
<td><em>Bifidobacterium breve</em> Yakult</td>
<td><em>B. breve</em> Yakult is still alive when it arrives in our intestines. It helps increase good bacteria and decrease bad ones. This improves the gastrointestinal environment and keeps the intestines healthy.</td>
</tr>
</tbody>
</table>


Based on submitted data. To emphasize that bifidobacteria and lactobacilli increase by using a particular product, it is necessary to conduct a study of the relevant product with humans and confirm the actual increase. Similarly, to emphasize that probiotics improve the intestinal environment or maintain good gastrointestinal conditions, it is necessary to present data from a study in humans to support the claim.


32.5 Korea
Lactic acid bacteria have been used for the manufacture of general foods, health functional foods, and pharmaceuticals in Korea. Generally, lactic acid bacteria found in the fermented foods are considered to be edible and hygienically safe. Among various general foods in Korea special food category containing lactic acid bacteria includes 'Lactic Acid Bacteria Drink' (LABD). The LABD drink should contain at least 1,000,000 CFU of viable form of lactic acid bacteria per milliliter of product. In Korea, general foods are prohibited to claim health function. The law governing the health function claim of the specific food is ‘Health Functional Food Act’ enacted by Korean Food and Drug Administration (KFDA) in 2003 by the Minister of Health and Welfare. The law was enacted to contribute to the improvement of national health and the consumer protection by ensuring safety, improving quality, and seeking sound distribution and sales of health functional food (KFDA). Under this law, the health functional food can claim for nutritional function, structure–function, and disease risk reduction function when the claim is supported by scientific evidences and approved by the KFDA. On the other hand, not only false or exaggerated labeling or advertising, but also claiming for the food product being efficient and effective in preventing and curing disease are prohibited. Generally, the manufacturing and quality of health functional food are required to be performed based on “GMP.”

Any person who intends to label and advertise the functionality of health functional food shall be deliberated in accordance with the standards of labeling and advertising deliberation of health functional food, and the methods and procedures thereof decided by the Commissioner of the FDA. The Minister of Health and Welfare established ‘The Health Functional Food Deliberation Committee’ to deliberate on the matters relating to the standards and specifications for health functional food and the labeling and advertising of health functional food.

32.5.1 Korean Health Functional Food Act

32.5.1.1 Article 1 (Purpose)
The purpose of this Act is to contribute to the improvement of national health and consumer protection by ensuring safety, improving quality, and seeking sound distribution and sales of health functional food.

32.5.1.2 Article 18 (Prohibition of False or Exaggerated Labeling and Advertising)
With respect to name, raw materials, manufacturing method, nutrients, components, usage method, quality, etc. of health functional food, no business person shall make false or exaggerated labeling or advertising falling under any of the following subparagraph: Labeling or advertising which may cause to mislead or confuse that it is efficient and effective in preventing and curing disease, or it is a medicine.

32.5.1.3 Article 22 (Good Manufacturing Practices, etc.)
For the purpose of manufacturing and quality control of good health functional food, the Commissioner of the FDA may determine and publicly announce the standards of manufacturing and quality control of good health functional food (hereinafter referred to as “GMP”).
3.2.5.1.4 Article 27 (Health Functional Food Deliberation Committee)

1. The Health Functional Food Deliberation Committee shall be established in the Ministry of Health and Welfare to research and deliberate on the following matters at the request of the Minister of Health and Welfare or the Commissioner of the FDA:
   a. Matters relating to the policies on health functional food

<table>
<thead>
<tr>
<th>Table 32.6</th>
<th>Standards and Specifications of the Probiotics in Korea</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Manufacturing standards</td>
<td></td>
</tr>
<tr>
<td>a. Raw material:</td>
<td></td>
</tr>
<tr>
<td>The microorganism itself or mixed</td>
<td></td>
</tr>
<tr>
<td><strong>Genus</strong></td>
<td><strong>Species</strong></td>
</tr>
<tr>
<td><em>Lactobacillus</em></td>
<td><em>L. acidophilus, L. casei, L. gasseri, L. delbrueckii ssp. bulgaricus,</em></td>
</tr>
<tr>
<td></td>
<td><em>L. helveticus, L. fermentum, L. paracasei, L. plantarum, L. reuteri,</em></td>
</tr>
<tr>
<td></td>
<td><em>L. rhamnosus, L. salivarius</em></td>
</tr>
<tr>
<td><em>Lactococcus</em></td>
<td><em>Lc. lactis</em></td>
</tr>
<tr>
<td><em>Enterococcus</em></td>
<td><em>E. faecium, E. faecalis</em></td>
</tr>
<tr>
<td><em>Streptococcus</em></td>
<td><em>S. thermophilus</em></td>
</tr>
<tr>
<td><em>Bifidobacterium</em></td>
<td><em>B. bifidum, B. breve, B. longum, B. animalis spp. lactis</em></td>
</tr>
<tr>
<td>b. Manufacturing method: It shall be edible form by culturing and then drying the microorganisms</td>
<td></td>
</tr>
<tr>
<td>c. Content of functional component (or biomarker): Alive bacteria shall be 100,000,000 CFU/g or more</td>
<td></td>
</tr>
<tr>
<td>2. Specifications</td>
<td></td>
</tr>
<tr>
<td>a. Appearance: It shall keep natural color, gloss, flavor, no off-taste, and off-flavor</td>
<td></td>
</tr>
<tr>
<td>b. Probiotics number: No less than labeled value</td>
<td></td>
</tr>
<tr>
<td>c. Coliform: Negative</td>
<td></td>
</tr>
<tr>
<td>3. Requisites of final product</td>
<td></td>
</tr>
<tr>
<td>a. Health claims: Help to maintain healthy gastrointestinal bacterial population; help to maintain healthy bowel function</td>
<td></td>
</tr>
<tr>
<td>b. Daily intake value: 100,000,000–10,000,000,000 CFU</td>
<td></td>
</tr>
<tr>
<td>4. Testing methods</td>
<td></td>
</tr>
<tr>
<td>a. Probiotics number</td>
<td></td>
</tr>
<tr>
<td>(i) <em>Lactobacillus</em> and -coccus: III.3.8.1 <em>Lactobacillus</em> and -coccus</td>
<td></td>
</tr>
<tr>
<td>(ii) <em>Bifidobacterium</em>: III.3.8.2 <em>Bifidobacterium</em></td>
<td></td>
</tr>
<tr>
<td>b. Coliform: Referred to the Annex of the Korean Health Functional Food Act</td>
<td></td>
</tr>
</tbody>
</table>
2. The Health Functional Food Deliberation Committee may appoint research fellows to research and study on the standards and specifications, the labeling and advertising of health functional food, etc.

3. Matters necessary for the organization and operation of the Health Functional Food Deliberation Committee as referred to in paragraphs (a) and (b) shall be prescribed by the Presidential Decree.

32.5.2 Regulations on Probiotics in Korea

Probiotics is one of the principle functional ingredients of health functional food approved by the KFDA. In Korea, acquisition of the functional food status for probiotics was supported by human clinical studies based on double-blind, randomized, placebo-controlled design using human-originated probiotic strains such as *Bifidobacterium bifidum* BGN4. The approved functional claims of the probiotics include: “Help to maintain healthy gastrointestinal bacteria population” and “Help to maintain healthy bowel function.” The standards and specifications of the probiotics are described in Table 32.6.

However, the intake of heterofermentative strains over the dose of suggested dosages was cautioned to cause gas flatulence and diarrhea. Additionally, the intake of the antibiotics together with probiotics was noted to inhibit the health function of the probiotics.

32.6 Malaysia

The food industry in Malaysia is under constant pressure to produce new and innovative foods by including food additive or supplements, for instance, probiotic and prebiotic, to enhance nutrition, nutrient function properties, as well as health and wellness benefits of food produced. However, to date, the Malaysian government has not yet developed extensive specific regulation for probiotic and prebiotic products in Malaysia. Nevertheless, there has been increased interest and efforts of the Food Safety and Quality Division (FSQD) of the Malaysian Ministry of Health (MOH) to improve regulatory control of nutrition labeling and health claims on probiotic and prebiotic, to ensure that such information are accurate and appropriate for the benefit of consumers.

The principal food law of Malaysia is under the authority of FSQD, which has been developed based on the Food Act 1983 (http://fsis2.moh.gov.my/fosimv2/HOM/frmHOMFARSec.aspx?id=22) and the Food Regulations 1985 (http://fsis2.moh.gov.my/fosimv2/HOM/frmHOMFARSec.aspx?id=21). Malaysia adopts a “positive list” approach, where only claims listed are permitted on food product. For food products regulated by FSQD, no claims can be made other than those permitted by the Food Regulations 1985.

Applications from the food industry for approval of a nutrient function claim must be accompanied by supporting documents, including results from five clinical trials and approval by other developed countries. The clinical trial results will be reviewed before recommendations are made by the Working Group on Nutrition and Health Claims and put forward for the consideration for endorsement by the Technical and Advisory Committee of Food Regulations.
32.6.1 Definition of Nutrition and Health Claims

The following is the summary of the definition of nutrition and health claims according to Codex Alimentarius, an international food standards setting organization.

Nutrition claim means any representation which states, suggests, or implies that a food has particular nutritional properties. There are two types of nutrition claims, namely, (1) nutrient content claim and (2) comparative claim. The Malaysian government enforces nutrient content claim and comparative claim with format and criteria generally similar to Codex Alimentarius.

Health claim means any representation that states, suggests, or implies that a relationship exists between a food or a constituent of food and health. These include: (1) nutrient function claim, (2) other function claim, and (3) reduction of disease risk claim. “Nutrient function claim” describes the physiological role of claimed nutrient on growth, development, and normal functions of the body. “Other function claim” describes specific beneficial effects of the consumption of a food constituent (or bioactive compound) in improving or modifying a physiological function. Particularly, probiotics and prebiotics are under the regulation of “other function claim.” “Reduction of disease risk claim” relates the consumption of a food or food constituent (bioactivities) with the reduced risk of developing a disease or health-related condition. However, “reduction of disease risk claim” is not permitted in Malaysia.

32.6.2 Health Claims Approved by Malaysian Ministry of Health

To date, 25 of “other function claims” for non-nutrients or food constituent or bioactive components have been reported in an official guidelines published by the FSQD of the MOH Malaysia (http://www.afic.org/labelingregulations.php?news_id=1120&start=0&category_id=43&parent_id=43&arcyear=&arcmonth). The claims were the petitions made by the industry. The “other function claims” that particularly related to probiotic and prebiotic are summarized in Table 32.7.

According to the Malaysian Food Regulation 1985 of Part V (Food Additives and Nutrient Supplement Bifidobacteria) and Part VII (Standards and Particular Labeling Requirement for Food Cultured Milk or Fermented Milk), bifidobacteria and other lactic acid bacteria are permitted to be used as food additive, nutrient supplement, or preparation of food cultured milk or fermented milk. Thus, the term “contain bifidus” or “with bifidus” may be used on the label of any package of food containing bifidobacteria. When a claim is made as to the presence of bifidobacteria in food, there shall be written in the label of a package containing such food, a statement setting out the viable bifidobacteria count present in a stated quantity of the food. There shall be written in the label on the package of food containing bifidobacteria the words “CONTAINS VIABLE BACTERIA, REQUIRE SPECIAL STORAGE CONDITION,” or “CONTAINS VIABLE BACTERIA, FOLLOW INSTRUCTION FOR STORAGE.”

As for cultured milk or fermented milk, for instance, yogurt, cultured cream, or sour cream shall be prepared by culturing pasteurized milk, sterilized milk, skimmed milk, recombined milk, pasteurized cream, or reduced cream with suitable lactic acid bacteria. They shall have an acidity of not less than 0.5% calculated as lactic acid and shall be written in the label on a package containing fermented milk the words “FERMENTED MILK.”
### Table 32.7 “Other Function Claims” That Are Particularly Related to Probiotics and Probiotic Products as Published in the Official Guidelines of the FSQD of the Malaysian MOH

<table>
<thead>
<tr>
<th>Food Constituent</th>
<th>Function Claim(s)</th>
</tr>
</thead>
</table>
| **1. Probiotic:** *Bifidobacterium lactis* | a. Helps to improve beneficial intestinal microflora  
   b. May help to reduce the incidence of diarrhea |
| **2. Prebiotic:** High amylose maize resistant starch | Helps to improve/promote colonic/bowel/intestinal function/environment |
| Inulin and oligofructose (fructo-oligosaccharide) | a. Inulin helps increase intestinal bifidobacteria and helps maintain a good intestinal environment  
   b. Oligofructose (fructo-oligosaccharide) helps increase intestinal bifidobacteria and helps maintain a good intestinal environment  
   c. Inulin is bifidogenic  
   d. Oligofructose (fructo-oligosaccharide) is bifidogenic  
   e. Inulin is probiotic  
   f. Oligofructose (fructo-oligosaccharide) is prebiotic |
| Oligosaccharide mixture containing 90% (wt/wt) GOS and 10% (wt/wt) lcFOS | a. Probiotic  
   b. Bifidogenic  
   c. Helps to increase intestinal bifidobacteria and help to maintain a good intestinal environment  
   d. Helps to improve the gut/intestinal immune systems of babies/infants |
| Oligofructose–inulin mixture containing 36%–42% oligofructose (DP 2–10) and 50%–56% inulin (DP > 10) | Helps to increase calcium absorption and increase bone mineral density when taken with calcium-rich foods |
| Polydextrose | a. Bifidogenic  
   b. Helps to increase intestinal bifidobacteria and helps maintain a good intestinal microflora |
| Resistant dextrin/Resistant maltodextrin | A soluble dietary fiber that helps to regulate/promote regular bowel movement especially of people with a tendency to constipation |

**Note:** GOS, Galacto-oligosaccharides; lcFOS, long-chain fructo-oligosaccharides; DP, degrees of polymerization.
32.7 Philippines

The FDA which was formerly referred to as the Bureau of Food and Drugs (BFAD) located at Filinvest Corporate City, Alabang, Muntinlupa City, Philippines, had issued Circular No. 16 s. 2004 signed by its Director, Prof. Leticia-Barbara B. Gutierrez on October 26, 2004. This circular refers to the guidelines on probiotics. Information that pertains to probiotics are stated in the circular. The BFAD informs the public, clients, and the BFAD personnel regarding guidelines on PROBIOTICS.

32.7.1 Guidelines on Probiotics

The term “Probiotic,” as defined by Gaarner and Scharfema in 1998 (as cited in Bureau Circular No. 16 s. 2004, BFAD, Philippines), was defined as “a dietary supplement based on living microorganisms which when administered in sufficient quantity, has a beneficial effect on the host organism, improving the equilibrium of the intestinal microflora.” The approved bacterial strains used as probiotics are as follows: *Lactobacilli*, *Bifidobacteria*, nonpathogenic strains of *Streptococcus*, *Saccharomyces boulardi*, and *Bacillus caussii*.

The use of bacterial strains not found in the above list shall be subject to (1) demonstration of evidence of safe use as food supplement and (2) analysis of the bacterial species found in the formulation. Likewise, BFAD shall use as reference FAO/WHO “Guidelines for the Evaluation of Probiotics in Food 2002.”

The BFAD informed everyone concerned that, for a Probiotic to be effective, the following properties should be demonstrated:

1. Beneficial effect on the host organism
2. Should be able to survive in the digestive tract
3. Should adhere to the mucosal epithelial cells
4. Should exhibit enhancement and protection of the intestinal ecology
5. Should remain viable during periods of storage and use

For the demonstration of the safety of a probiotic, the following documents should be submitted:

1. Determination of antibiotic resistance patterns.
2. Assessment of certain metabolic activities (e.g., d-lactate production, bile salt deconjugation).
3. Assessment of side-effects during human studies.
4. Epidemiological surveillance of adverse incidents in consumers (post-market).
5. If the strain under evaluation belongs to a species that is a known mammalian toxin producer, it must be tested for toxin production. One possible scheme for testing toxin production has been recommended by the EU Scientific Committee on Animal Nutrition (SCAN 2000).
6. If the strain under evaluation belongs to a species with known hemolytic potential, determination of hemolytic activity is required.

32.7.2 Probiotic Claims

BFAD recognizes and approves the following probiotic claims as food supplements:

1. Enhancement of intestinal ecology
2. Helping improve lactose malabsorption
3. Improving digestion
4. Aid to the enhancement of natural resistance to intestinal infections

Such claims may be reflected on the labels and can be used for advertisement and product promotion.

When a probiotic makes a claim of altering disease or as an immunomodulator, the said product shall be classified as a drug. Such above-cited claims denote therapeutic advantage that only a drug product can make. The therapeutic claim should be based on sound scientific evidence based on studies on human subjects.

32.7.3 Guidelines for the Evaluation of Probiotics for Drug Use (Bureau Circular No. 16 s. 2004 of BFAD, Philippines)

For guidelines for the evaluation of probiotic for drug use, see Figure 32.2.

Figure 32.2  Guidelines for the evaluation of probiotics for drug use in the Philippines.
32.7.4 Guidelines for the Evaluation of Probiotic Food Use  
*(Bureau Circular No. 16 s. 2004 of BFAD, Philippines)*

For guidelines for the evaluation of probiotic food use, see Figure 32.3.

32.8 Singapore

Probiotics may be used in Food and Health Supplements. Health supplements are currently not subject to approvals and licensing by Health Science Authority before they can be marketed in Singapore. However, dealers of health supplements, including probiotic products, are accountable for ensuring that their products do not contain prohibited ingredients, synthetic drugs, or toxic heavy metals above legally permissible limits.

The Agri-Food and Veterinary Authority (AVA) has neither published a list nor a recommended dosing of probiotics-based health supplement products. Dealers are responsible for the safety and quality of the product they produce and sell.

In principle, all ingredients and additives used in food must be safe, and probiotics are not excluded. AVA has allowed strains of bifidobacteria and lactobacillus that have a proven long history of safe use in food (such as *B. bifidum*, *L. acidophilus*, *L. delbrueckii*, and *L. casei* Shirota strain) to be used as probiotics in suitable categories of food products.
As in the case of major developed countries, Singapore does not set levels for the minimum and maximum quantities of probiotic bacteria allowed to be added to food. Instead, manufacturers should ensure that the quantities of probiotic bacteria present in their food products are safe and suitable for consumption by the target group of consumers, and are sufficient to deliver the intended function as claimed.

The following nutrient function claims relating to the role of probiotics (exact name of probiotic must be specified) in helping to maintain a healthy digestive system through suppressing the growth of harmful bacteria may be used, as long as they are truthful and can be substantiated. Applications for use of new nutrient function claims may be considered by AVA on a case-by-case basis. The following are some examples approved.

1. Helps to maintain a healthy digestive system
2. Helps in digestion
3. Helps to maintain a desirable balance of beneficial bacteria in the digestive system
4. Helps to suppress/fight against harmful bacteria in the digestive system, thereby helping to maintain a healthy digestive system

### 32.9 Taiwan

At present, the Taiwan government focuses very intensely on food safety, especially on both chemical and microbiological hazards. The Department of Health (DOH) is the statutory body responsible for the management of food safety in Taiwan. Food products must comply with the “Law Governing Food Sanitation,” together with its enforcement rules and a series of food standards promulgated by the DOH. The term “foods” as used in this law refers to goods provided to people for eating, drinking, or chewing, and their raw materials (DOH, Executive Yuan, Taiwan, 2010). In Taiwan, LAB strains used in food can be classified into two categories according to their functions. The first category acts as ingredients or processing aid for food. The second category involves the probiotic functions.

#### 32.9.1 Regulating the Use of LAB as Ingredients or Processing Aid for Food

The DOH in Taiwan lists all the raw materials that could be used in food products including microorganisms (Table 32.8). The raw materials in the list require no further toxicity test if they satisfy one of the following two conditions.

1. The raw materials of the product are conventional foodstuff and are usually consumed as processed food.
2. There is a complete academic literature report on the toxicity safety of the product and a record of human consumption; the raw materials, composition of ingredients and manufacturing procedure of the product are completely in line with the findings stated in the academic literature report submitted.

The list includes the common LAB genera in food fermentations, such as *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Bifidobacterium*, and *Streptococcus*. Of these, *Lactobacillus*, *Leuconostoc*, and *Streptococcus*...
### Table 32.8  List of Microorganisms Allowed in Food Products Bulletin by the Department of Health

<table>
<thead>
<tr>
<th>No.</th>
<th>Type</th>
<th>Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Lactic acid bacteria</td>
<td><em>Bacillus coagulans</em></td>
</tr>
<tr>
<td>2</td>
<td></td>
<td><em>Bacillus mesentericus</em></td>
</tr>
<tr>
<td>3</td>
<td></td>
<td><em>Bacillus natto, Bacillus subtilis</em></td>
</tr>
<tr>
<td>4</td>
<td></td>
<td><em>Bifidobacterium adolescentis</em></td>
</tr>
<tr>
<td>5</td>
<td>Lactic acid bacteria</td>
<td><em>Bifidobacterium bifidum</em></td>
</tr>
<tr>
<td>6</td>
<td>Lactic acid bacteria</td>
<td><em>Bifidobacterium breve</em></td>
</tr>
<tr>
<td>7</td>
<td>Lactic acid bacteria</td>
<td><em>Bifidobacterium infantis</em></td>
</tr>
<tr>
<td>8</td>
<td>Lactic acid bacteria</td>
<td><em>Bifidobacterium lactis</em></td>
</tr>
<tr>
<td>9</td>
<td>Lactic acid bacteria</td>
<td><em>Bifidobacterium longum</em></td>
</tr>
<tr>
<td>10</td>
<td></td>
<td><em>Clostridium butyricum</em></td>
</tr>
<tr>
<td>11</td>
<td>Lactic acid bacteria</td>
<td><em>Enterococcus faecalis</em></td>
</tr>
<tr>
<td>12</td>
<td>Lactic acid bacteria</td>
<td><em>Enterococcus faecium (Streptococcus faecium)</em></td>
</tr>
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<td>13</td>
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<td><em>Lactobacillus acidophilus</em></td>
</tr>
<tr>
<td>14</td>
<td>Lactic acid bacteria</td>
<td><em>Lactobacillus bifidus</em></td>
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<tr>
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<td><em>Lactobacillus brevis</em></td>
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<td>16</td>
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<td><em>Lactobacillus bulgaricus</em></td>
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<td><em>Lactobacillus casei</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Lactobacillus casei subsp. rhamnosus</em></td>
</tr>
<tr>
<td>18</td>
<td>Lactic acid bacteria</td>
<td><em>Lactobacillus cremoris</em></td>
</tr>
<tr>
<td>19</td>
<td>Lactic acid bacteria</td>
<td><em>Lactobacillus delbrueckii</em></td>
</tr>
<tr>
<td>20</td>
<td>Lactic acid bacteria</td>
<td><em>Lactobacillus delbrueckii subsp. bulgaricus</em></td>
</tr>
<tr>
<td>21</td>
<td>Lactic acid bacteria</td>
<td><em>Lactobacillus fermentum</em></td>
</tr>
<tr>
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<td><em>Lactobacillus gasseri</em></td>
</tr>
<tr>
<td>23</td>
<td>Lactic acid bacteria</td>
<td><em>Lactobacillus helveticus</em></td>
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<td>Lactic acid bacteria</td>
<td><em>Lactobacillus kefir</em></td>
</tr>
<tr>
<td>25</td>
<td>Lactic acid bacteria</td>
<td><em>Lactobacillus lactis</em></td>
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<tr>
<td>26</td>
<td>Lactic acid bacteria</td>
<td><em>Lactobacillus lactis subsp. lactis</em></td>
</tr>
<tr>
<td>27</td>
<td>Lactic acid bacteria</td>
<td><em>Lactobacillus paracasei</em></td>
</tr>
<tr>
<td>28</td>
<td>Lactic acid bacteria</td>
<td><em>Lactobacillus plantarum</em></td>
</tr>
</tbody>
</table>

(continued)
Table 32.8  List of Microorganisms Allowed in Food Products Bulletined by the Department of Health (Continued)

<table>
<thead>
<tr>
<th>No.</th>
<th>Type</th>
<th>Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>29</td>
<td>Lactic acid bacteria</td>
<td>Lactobacillus reuteri</td>
</tr>
<tr>
<td>30</td>
<td>Lactic acid bacteria</td>
<td>Lactobacillus rhamnosus</td>
</tr>
<tr>
<td>31</td>
<td>Lactic acid bacteria</td>
<td>Lactobacillus salivarius</td>
</tr>
<tr>
<td>32</td>
<td>Lactic acid bacteria</td>
<td>Lactobacillus sporogenes</td>
</tr>
<tr>
<td>33</td>
<td>Anka</td>
<td>Monascus anka, Monascus berkeri, Monascus pilosus,</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Monascus purpureus, Monascus ruber</td>
</tr>
<tr>
<td>34</td>
<td>Lactic acid bacteria</td>
<td>Propionibacterium freudenreichii, Propionibacterium</td>
</tr>
<tr>
<td></td>
<td></td>
<td>shermanii</td>
</tr>
<tr>
<td>35</td>
<td>Beer yeast, Brewer’s yeast</td>
<td>Saccharomyces cerevisiae</td>
</tr>
<tr>
<td>36</td>
<td>Zinc yeast</td>
<td>Saccharomyces cerevisiae</td>
</tr>
<tr>
<td>37</td>
<td>Selenium yeast</td>
<td>Saccharomyces cerevisiae</td>
</tr>
<tr>
<td>38</td>
<td>Chromium yeast</td>
<td>Saccharomyces cerevisiae</td>
</tr>
<tr>
<td>39</td>
<td>Iron yeast</td>
<td>Saccharomyces cerevisiae</td>
</tr>
<tr>
<td>40</td>
<td>Magnesium yeast</td>
<td>Saccharomyces cerevisiae</td>
</tr>
<tr>
<td>41</td>
<td>Potassium yeast</td>
<td>Saccharomyces cerevisiae</td>
</tr>
<tr>
<td>42</td>
<td>Molybdenum yeast</td>
<td>Saccharomyces cerevisiae</td>
</tr>
<tr>
<td>43</td>
<td>Yeast</td>
<td>Saccharomyces fragilis, Kluyveromyces fragilis</td>
</tr>
<tr>
<td>44</td>
<td>Lactic acid bacteria</td>
<td>Sporolactobacillus inulinus</td>
</tr>
<tr>
<td>45</td>
<td>Lactic acid bacteria</td>
<td>Streptococcus lactis</td>
</tr>
<tr>
<td>46</td>
<td>Lactic acid bacteria</td>
<td>Streptococcus salivarius subsp. thermophilus</td>
</tr>
<tr>
<td>47</td>
<td>Lactic acid bacteria</td>
<td>Streptococcus thermophilus</td>
</tr>
<tr>
<td>48</td>
<td>Yeast</td>
<td>Saccharomyces cerevisiae ssp. chevalieri</td>
</tr>
<tr>
<td>49</td>
<td>Lactic acid bacteria</td>
<td>Lactobacillus pentosus</td>
</tr>
<tr>
<td>50</td>
<td>Lactic acid bacteria</td>
<td>Streptococcus faecalis</td>
</tr>
<tr>
<td>51</td>
<td>Yeast</td>
<td>Candida utilis</td>
</tr>
<tr>
<td>52</td>
<td>Lactic acid bacteria</td>
<td>Lactococcus lactis subsp. cremoris</td>
</tr>
<tr>
<td>53</td>
<td>Lactic acid bacteria</td>
<td>Lactococcus lactis subsp. lactis</td>
</tr>
<tr>
<td>54</td>
<td>Lactic acid bacteria</td>
<td>Lactococcus lactis subsp. lactis biovar. diacetylactis</td>
</tr>
<tr>
<td>55</td>
<td>Lactic acid bacteria</td>
<td>Leuconostoc mesenteroides subsp. cremoris</td>
</tr>
</tbody>
</table>

Source: Department of Health, Executive Yuan, Taiwan, 2009.
historically represent the major genera in the fermented dairy products, such as yogurt and cheese. The only species of genus *Streptococcus* in the list associated with food fermentations is *S. thermophilus*.

*Bifidobacterium* fermented products do not possess the typical desirable flavor associated with yogurt or other dairy products due to the acetic acid production (Lourens-Hattingh and Viljoen 2001). However, the bifidobacteria group is considered a “probiotic.” Bifidobacteria are one of the hundreds of beneficial bacteria that inhabit the body’s intestinal tract. *Bifidobacterium* is better to culture along with other lactic acid bacteria to improve the flavor (Modler 1994).

*Bac. coagulans* has also been assessed for safety as a food ingredient by the DOH. *Bac. coagulans*, initially considered to be a spore-forming *Lactobacillus*, is a lactic acid-forming bacterial species within the genus *Bacillus*. This strain is often marketed as *L. sporogenes* as probiotics in improving the vaginal flora, and improving abdominal pain and bloating in Irritable Bowel Syndrome patients (Sanders et al. 2003).

For importing microorganisms as food ingredients in the DOH list, besides requiring the Taiwan’s food import regulations, the following files should be documented for the DOH approval.

1. (1) Taxonomy report including scientific name and authority, history of cultures, characteristics of cultures, and pathogenic effects
2. Report of laboratory testing for the purity of the microorganism
3. The photocopy of the import registration license
4. The name and address of the manufacturer

Most information regarding Taiwan’s food import regulations is available on the internet at: http://www.doh.gov.tw. If the LAB strains are not on the list (Table 32.8), the DOH must first be notified of the new culture before its marketing, and the strain’s safety and efficacy must be documented.

In Taiwan, for registration of new raw materials used in food products including microorganisms, the following documents and materials are required for application of DOH approval before manufacturing and marketing.

1. An application form including:
   a. The original copy and its duplicate of reports on ingredient list, product specification, and nutrient analysis. The original manufacturer shall issue these reports within 1 year.
   b. The ingredient list report shall specify detailed composition of raw materials and food additives.
   c. A copy of a summarized diagram on the manufacturing process.
   d. Microorganism should include taxonomy (scientific name and authority, history of cultures), characteristics of cultures, and safety aspects.
   e. The ingredient list shall specify the Recommended Daily Dosage of the product.
2. An official certificate attesting to the legitimacy of the original manufacturer.
3. A duplicate copy of the business license of the applicant.

### 32.9.2 Regulating the Use of LAB as Probiotics

1. Health Food Control Act
   The LAB strains claiming health benefits should follow the “Health Food Control Act” in Taiwan. The “Health Food Control Act” was first promulgated on February 3, 1999, and amended on May 17, 2006. In the Act, health food is defined as “food with specific nutrient or health maintenance effects which is especially labeled or advertised, and do not aim at
Probiotics Regulation in Asian Countries

“Health maintenance effects” are effects recognized by the DOH as those which promote the health of citizens or reduce the risk of serious illnesses. So far, the DOH has given recognition to 10 such effects, namely:

a. Regulation of blood lipid
b. Promotion of gastrointestinal functions
c. Alleviation of osteoporosis
d. Maintenance of dental health
e. Regulation of the immune system
f. Regulation of blood sugar level
g. Protection of the liver
h. Postpone aging
i. Anti-weakness
j. Regulating blood pressure

These effects are not therapeutic such as treating or remediying human diseases. Health food also includes food in tablet, capsule, powder, or oral liquid forms. According to the DOH, products with health food claims must prove that they possess the ability to contribute to the health of those consuming them. Any product claiming to be a health food must receive the DOH’s approval before being marketed. Figure 32.4 shows the flowchart on application for a health food permit. There are two tracks of application. If the products satisfy the requirements of the special item standards (so far, only for fish oil and anka products), the following reports are no longer needed: safety assessment report, health care effect assessment report, and relevant research reports and literatures (Track 2). Otherwise, the product should follow the flowchart in the Track 1. The information regarding Health Food Control Act is available on the internet at: http://www.doh.gov.tw.

When the products are approved by DOH as health food, the following information, in Chinese and commonly used symbols, shall be conspicuously displayed on the containers, packaging, or written instructions of the health foods:

a. Product name
b. Name and weight or volume of the contents (if a mixture of two or more components, they must be listed separately)
c. Name of food additives
d. Expiry date, method, and conditions of preservation
e. Name and address of the responsible business operator; the name and address of the importer shall be specified if the health food is imported
f. The approved health care effects
g. Reference number of the permit, the legend of “health food” and standard logo
h. Intake amount and other important messages for the consumption of the health food along with other necessary warnings
i. Nutrient and its content
j. Other material facts designated by the DOH
k. Country of origin

2. Health food related to lactic acid bacteria

Up to now, among 187 food products obtaining the health food permits authorized by the DOH, 41 items are lactic acid bacteria-related products (Table 32.9). The dairy-related
The flowchart of application for health food permit

Figure 32.4 Flowchart for application of health food permit. (From Department of Health, Executive Yuan, Taiwan, 2009.) Figure 32.4a and b represent tracks 1 and 2, respectively.
products such as drinking yogurts (19 items), milk powders (3 items), stirred yogurt (1 item), and fresh milk (1 item) are the major food types. However, LAB products in the powder, tablet, and capsule forms are also getting popular in Taiwan market due to their easy consumption and preservation.

Among the 41 LAB-related products, the most common strains associated with health food products are *L. acidophilus*, *L. casei*, *L. delbrueckii*, *L. paracasei*, *B. lactis*, *B. longum*, and *S. thermophilus*. Several other lactic acid bacteria, such as *L. sporogenes*, *L. fermentum*, *L. johnsonii*, *L. salivarius*, *L. gasseri*, and *Bac. coagulans* are also utilized in the health products. The strains used in the 41 LAB-related products all appear on the list of microorganisms bulletined by DOH. The safety assessment of these LAB strains is not required. However, the safety assessment of *L. rhamnosus* Tcell-1 in TCELL-1LAB powder, including genotoxicity test and 28-day feeding toxicity test, is provided by the manufacturer.

Considering the functional properties linked to lactic acid bacteria, all items fall into the following four functional categories.

1. Promotion of gastrointestinal functions
   This category is the most common functional property associated with LAB. Thirty-three of the 41 LAB-related products are associated with promotion of gastrointestinal functions specified in assistance of increasing population of intestinal probiotic microflora, limited the growth of *Clostridium perfringens*, and keeping the balance of intestinal microflora. The LAB strains involving in promotion of gastrointestinal functions mostly belong to genus *Lactobacillus* (*L. delbrueckii*, *L. acidophilus*, *L. paracasei*, *L. rhamnosus*, and *L. fermentum*) and *Bifodobacterium* (*B. longum*, *B. lactis*, and *B. bifidum*). Other species such as *S. thermophilus* and *Bac. coagulans* are also included in this category.

   It is worth mentioning that the AB drinking yogurt manufactured by UniPresident also claims the ability to inhibit *Helicobacter pylori* growth (Table 32.9). LAB are thought to aid in the treatment of *H. pylori* infections (which cause peptic ulcers) in adults when used in combination with standard medical treatments (Eaton et al. 1991; Hamilton-Miller 2003).

2. Regulation of immune system
   Five of the 41 LAB-related products claimed the property of regulation of the immune system mainly in anti-allergic effects. Many studies have examined the efficacy of LAB supplementation in the immunoregulation (Hong et al. 2009). Although the detailed mechanisms of these effects exerted by LAB have not yet been clarified, cytokines induced by LAB are considered to play key roles in immunoregulation (Shida et al. 2006).

   To evaluate the anti-allergic effects of LAB, oral administration of LAB-related products in ovalbumin (OVA) sensitized mice was conducted. The abilities of enhancing the production of T helper (Th) 1 cytokines (interferon (IFN)-γ, interleukin (IL)-2) and inhibiting the secretion of Th2 cytokines (IL-4, IL-5, IL-10) were determined (Matsuzaki et al. 1998). The effect of suppressing serum IgE and IgG1 secretion in a food allergy model was also investigated for LAB anti-allergic effects (Shida et al. 2002).

   The LAB strains involved in the regulation of the immune system all belong to genus *Lactobacillus* including *L. salivarius* subsp. salicinius, *L. johnsonii* EM1, *L. paracasei* 33, *L. casei* Shirota, and *L. salivarius* PM-A006.

3. Regulation of blood lipid
   Only 1 LAB product (Quaker high calcium nonfat milk powder) received the health food permit issued by the DOH in regulation of blood lipid specified in lowering both triglyceride
<table>
<thead>
<tr>
<th>Product Brand</th>
<th>Type</th>
<th>LAB Strains</th>
<th>Health Benefits</th>
</tr>
</thead>
<tbody>
<tr>
<td>UniPresident AB drinking yogurt</td>
<td>Drinking yogurt</td>
<td><em>Lactobacillus acidophilus</em> (La-5) <em>Bifidobacterium lactis</em> (Bb-12)</td>
<td>Promotion of gastrointestinal functions 1. Increasing population of intestinal probiotic microflora 2. Inhibition of <em>Helicobacter pylori</em> growth</td>
</tr>
<tr>
<td>Bifidobacterium longum BB536</td>
<td>Powder</td>
<td><em>Bifidobacterium longum</em> BB536</td>
<td>Promotion of gastrointestinal functions 1. Increasing population of intestinal probiotic microflora</td>
</tr>
<tr>
<td>Complex probiotics</td>
<td>Powder</td>
<td><em>Bifidobacterium longum</em> BB536 <em>Bifidobacterium lactis</em> Bb12 <em>Lactobacillus acidophilus</em> <em>Lactobacillus casei</em></td>
<td>Promotion of gastrointestinal functions 1. Increasing population of intestinal probiotic microflora</td>
</tr>
<tr>
<td>Yoplait low fat plain</td>
<td>Drinking yogurt</td>
<td><em>Streptococcus thermophilus</em> <em>Lactobacillus delbrueckii</em></td>
<td>Promotion of gastrointestinal functions 1. Increasing population of intestinal probiotic microflora</td>
</tr>
<tr>
<td>Yoplait low fat plain with microencapsulated probiotics</td>
<td>Drinking yogurt</td>
<td><em>Streptococcus thermophilus</em> <em>Lactobacillus delbrueckii</em> <em>Bifidobacterium longum</em></td>
<td>Promotion of gastrointestinal functions 1. Increasing population of intestinal probiotic microflora</td>
</tr>
<tr>
<td>Yoplait strawberry with <em>Bifidobacterium longum</em></td>
<td>Drinking yogurt</td>
<td><em>Streptococcus thermophilus</em> <em>Lactobacillus delbrueckii</em> <em>Bifidobacterium longum</em> <em>Lactobacillus paracasei</em> <em>Lactobacillus acidophilus</em></td>
<td>Promotion of gastrointestinal functions 1. Increasing population of intestinal probiotic microflora</td>
</tr>
<tr>
<td>No.</td>
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<td>Formulation</td>
<td>Benefits</td>
</tr>
<tr>
<td>-----</td>
<td>----------------------------------------------------------</td>
<td>-------------------------------</td>
<td>-----------------------------------------------------------------------------------------------</td>
</tr>
</tbody>
</table>
| 7   | Kuang-Chuan low fat with microencapsulated probiotics   | Drinking yogurt             | *Bifidobacterium longum*  
1. Increasing population of intestinal probiotic microflora |
| 8   | King Car lactic acid bacteria                           | Powder and tablet            | *Bifidobacterium lactis*  
*Lactobacillus acidophilus*  
*Lactobacillus paracasei*  
1. Increasing population of bifidobacteria |
| 9   | Grape king lactic acid bacteria                          | Powder and tablet            | *Lactobacillus casei sp. rhamnosus GG*  
1. Increasing population of intestinal probiotic microflora |
| 10  | Quaker high calcium nonfat milk powder                  | Milk powder                  | *Lactobacillus acidophilus*  
*Lactobacillus casei*  
*Bifidobacterium lactis*  
1. Increasing population of intestinal probiotic microflora  
2. Effectively suppressing the growth of harmful bacteria in the intestine |
| 11  | Yakult                                                   | Drinking yogurt              | *Lactobacillus casei Shirota*  
1. Increasing population of intestinal probiotic microflora  
2. Effectively suppressing the growth of harmful bacteria in the intestine |
| 12  | Weichuan Lin phone high quality yogurt                  | Drinking yogurt              | *Streptococcus thermophilus*  
*Lactobacillus delbrueckii subsp. bulgaricus*  
*Lactobacillus acidophilus*  
*Bifidobacterium longum*  
1. Increasing population of intestinal probiotic microflora |

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<tr>
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<td>Milk Powder</td>
<td><em>Lactobacillus acidophilus</em></td>
<td>Promotion of gastrointestinal functions 1. Increasing population of intestinal probiotic microflora</td>
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<td>Powder</td>
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<td>15 Chang La Probiotics</td>
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<tr>
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<td><em>Lactobacillus sporogenes</em></td>
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<td>16 FloraGuard®</td>
<td>Beverage</td>
<td><em>Lactobacillus acidophilus</em></td>
<td>Promotion of gastrointestinal functions 1. Increasing population of intestinal probiotic microflora</td>
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<td><em>Lactobacillus bulgaricus</em></td>
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<td><em>Streptococcus thermophilus</em></td>
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<td>17 LGG Yogurt</td>
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<td></td>
<td><em>Bifidobacterium bifidum</em></td>
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<td>ProBio PCC</td>
<td>Capsule</td>
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<td>Yogurt with fruit</td>
<td>Stirred Yogurt</td>
<td><em>Streptococcus thermophilus</em> Lactobacillus delbrueckii subsp. bulgaricus</td>
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<td>Grape king probiotics</td>
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<td><em>Bifidobacterium longum</em></td>
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<td>22</td>
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<td>Milk powder</td>
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<th>LAB Strains</th>
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| 25 Yakult Blueberry high calcium yogurt | Drinking yogurt | *Lactobacillus casei* Shirota  
*S. thermophilus* | Promotion of gastrointestinal functions  
1. Increasing population of intestinal probiotic microflora |
| 26 Yakult Strawberry high calcium yogurt | Drinking yogurt | *Lactobacillus casei* Shirota  
*S. thermophilus* | Promotion of gastrointestinal functions  
1. Increasing population of intestinal probiotic microflora |
| 27 Yakult Blueberry high calcium yogurt | Drinking yogurt | *Lactobacillus casei* Shirota  
*S. thermophilus* | Promotion of gastrointestinal functions  
1. Increasing population of intestinal probiotic microflora |
| 28 Sint *Lactobacillus salivarius* capsules | Capsule | *Lactobacillus salivarius* PM-A006 | Regulation of the immune system  
1. Inhibition of the OVA-specific IgE production in serum  
2. Stimulation of IFN-γ production in spleen cells |
| 29 Yakult 300 light | Drinking yogurt | *Lactobacillus casei* Shirota | 1. Promotion of gastrointestinal functions  
a. Enhancement of activities of nature killer cells  
b. Enhancement of activities of phagocytic cells  
c. Increasing population of IgM and IgA in serum  
2. Regulation of the immune system  
a. Increasing population of intestinal probiotic microflora  
b. Limited the growth of *Clostridium perfringens* |
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<td>Drinking yogurt</td>
<td><em>Streptococcus thermophilus</em>&lt;br&gt;<em>Lactobacillus paracasei</em>&lt;br&gt;<em>Lactobacillus bulgaricus</em></td>
<td>Regulation of the immune system&lt;br&gt;1. Enhancement of activities of type I T helper cells</td>
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<tr>
<td>31</td>
<td>eN-Lac Plus Capsules</td>
<td>Capsule</td>
<td><em>Lactobacillus paracasei</em>&lt;br&gt;<em>Lactobacillus fermentum</em>&lt;br&gt;<em>Lactobacillus acidophilus</em></td>
<td>Regulation of the immune system&lt;br&gt;1. Inhibition of the OVA-specific IgE production in serum&lt;br&gt;2. Reducing the production of IL-5 in spleen cells</td>
</tr>
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<td>Dental Lac Troches</td>
<td>Tablet</td>
<td><em>Lactobacillus paracasei</em></td>
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<td>Kuangchuan fresh milk with probiotics</td>
<td>Milk</td>
<td><em>Bifidobacteria longum</em></td>
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<td>34</td>
<td>TTY BioPharmTen Billion probiotics</td>
<td>Tablet</td>
<td><em>Lactobacillus johnsonii</em>&lt;br&gt;EM1</td>
<td>Regulation of the immune system&lt;br&gt;1. Reducing the production of IL-5&lt;br&gt;2. Promoting the proliferation of spleen cells</td>
</tr>
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<td>35</td>
<td>Brands Xylooligo-Saccharides Plus LAB powder</td>
<td>Powder</td>
<td><em>Bacillus coagulans</em>&lt;br&gt;<em>Lactobacillus acidophilus</em></td>
<td>Promotion of gastrointestinal functions&lt;br&gt;1. Increasing population of intestinal probiotic microflora</td>
</tr>
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<td>36</td>
<td>LS99 functional drinking yogurt</td>
<td>Drinking yogurt</td>
<td><em>Lactobacillus salivarius</em>&lt;br&gt;subsp.&lt;br&gt;<em>salicinius</em></td>
<td>Regulation of the immune system&lt;br&gt;1. Inhibition of the OVA-specific IgE production in serum</td>
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<tr>
<th>Product Brand</th>
<th>Type</th>
<th>LAB Strains</th>
<th>Health Benefits</th>
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</table>
| **37 Wei-Jei Capsule**                | Capsules      | *Lactobacillus casei* sp. *rhamnosus*  
*Lactobacillus gasseri* | Promotion of gastrointestinal functions  
1. Inhibition of *Helicobacter pylori* growth |
| **38 Chlorella Plus Bacillus**        | Tablets       | *Bacillus coagulans*         | Promotion of gastrointestinal functions  
1. Increasing population of intestinal probiotic microflora |
| **39 UniPresident AB drinking yogurt (no sugar)** | Drinking yogurt | *Lactobacillus acidophilus* (La-5)  
*Bifidobacterium lactis* (Bb-12) | Promotion of gastrointestinal functions  
1. Increasing population of intestinal probiotic microflora |
| **40 Taisugarfructo-oligosaccharide and lactic acid bacteria** | Powder        | *Lactobacillus sporogenes*  
*Lactobacillus acidophilus* | Promotion of gastrointestinal functions  
1. Increasing population of intestinal probiotic microflora |
| **41 Yakult 300**                     | Drinking yogurt | *Lactobacillus casei* Shirota | Promotion of gastrointestinal functions  
1. Increasing population of intestinal probiotic microflora  
2. Limited the growth of *Clostridium perfringens* |
level and low density lipoprotein (LDL) level as well as increasing high-density lipoprotein (HDL) level. Several studies have demonstrated in animal model that a range of LAB is able to lower serum cholesterol levels. The possible mechanisms involved breaking down bile in the gut, thus inhibiting its re-absorption (which enters the blood as cholesterol). Some of human trials also demonstrated that dairy foods fermented with specific LAB can produce modest reductions in total and LDL cholesterol levels (Wollowski et al. 2001).

4. Maintenance of dental health

One item, Dental Lac Troches with *L. paracasei*, is associated with the property of maintaining dental health. The *L. paracasei* could limit the growth of *S. mutans*, which is a human odontopathogen. Loesche (1986) indicated that acid uricity appears to be the most consistent attribute of *S. mutans* that is associated with both its colonization at stagnant areas and its cariogenicity. Kohler et al. (1984) suggested that treatment strategies that interfere with the colonization of *S. mutans* may have a profound effect on the incidence of dental decay in human populations.

It is also interesting to note two trends in Taiwan health food products being marketed. The first one is that one health food item claims more than one functional properties. The second is the multiple probiotic strains used in one product. Research is emerging on the potential health benefits of multiple probiotic strains as a health supplement as opposed to a single strain. The human gut has more than 500 types of microbes. It is thought that this diverse environment may benefit from multiple probiotic strains. Different strains may also be associated with different health benefits. More consumers in Taiwan prefer the multiple functional products.

Most well-known lactic acid bacteria have a long and safe history of use in food. They are generally considered safe. However, other less-well-known LAB may require more specialized knowledge as they are isolated and introduced into foods. According to Taiwan food safety regulatory requirement, it is incumbent on the food industry to ensure that these newly discovered microbial food cultures are safe. Additionally, the LAB strains providing health benefits should follow the “Health Food Control Act” in Taiwan.

### 32.10 Thailand

Probiotic has come to Thailand as dairy products such as yogurt, drinking yogurt, and powder milk since 2001. To provide better information relating to its health claim to ensure standards and protect consumers, Thai FDA, Ministry of Public Health Thailand started regulating probiotic usage for food product since 2008 (FDA 403/2551) and it is summarized in the following.

#### 32.10.1 Scope

Regulation of probiotic is established by a committee composed of specialists from medical, nutritional, food biotechnology, and pharmaceutical fields. The committee agreed that probiotic is used in conventional food and dietary supplement manufactured locally or imported and sold. Health claims should exclude reference to the term bio-therapeutic agents, beneficial microorganisms in other non-food and genetically modified microorganisms.
32.10.2 Definition

*Probiotic* is defined according to WHO 2001 as “Live microorganisms which when administered in adequate amounts confer a health benefit on the host.” This definition restricted the use of the word probiotic to products which contain live microorganism and the need for providing an adequate dose to exert the desirable effects.

*Biomarker* is defined as various characteristics of probiotic, which are assessed as the index of normal biological process, pathology, and pharmaceutical response after ingestion of the probiotic. *Health Claim* refers to beneficial food or food composition or food components relating to health promotion either direct or indirect.

32.10.3 Guidelines for the Assessment of Probiotic Microorganisms

To claim a probiotic food, the probiotic assessment of quality, safety, and efficiency are needed. Their guidelines are mainly modified from Report of Joint FAO/WHO (2001, 2002).

32.10.3.1 Identification of Genus, Species, and Strain of Probiotic Microorganisms

Microorganisms having a probiotic property are strain specific. Determination of microbial strains and their effects to health and toxicity study are needed because probiotic properties are strain related. It is recommended that strain identification be performed using methodology with high accuracy and reliable. It is recommended that both phenotypic and genotypic tests should be conducted. The proposed probiotics should be named according to (1) International Code of Nomenclature which provides Approved Lists of Bacteria names (Skerman et al. 1980) and/or (2) Validation Lists, published in the International Journal of Systematic and Evolutionary Microbiology or International Journal of Systematic Bacteriology, before 2002 or Bergey’s Manual.

32.10.3.2 Selection of Probiotic Microorganisms

To assess the properties of probiotics, it is suggested that the following guidelines are needed. For food application, probiotic microorganisms should be able to survive passage through the digestive tract and to proliferate in the gut. The following *in vitro* tests are needed: (1) resistance to gastric acidity and bile acid, (2) adherence to mucus and/or human epithelial cells and cell line, (3) ability to reduce pathogen adhesion to surfaces, and (4) having bile salt hydrolase activity.

However, only *in vitro* tests are not enough to conclude probiotic property for humans. Further study on clinical tests is needed.

32.10.3.3 Safety Assessment of Probiotics to Humans

Lactobacilli and bifidobacteria are widely used in the food industry long ago and their uses have not been associated with disease. Therefore, it is concluded that these two bacterial groups are considered to be safe and allowed to be used as probiotics. Even though they are classified as GRAS, some rare cases of *Lactobacillus* and *Bifidobacterium* infection can cause side-effects, which could be classified into four types: (1) systemic infections, (2) deleterious metabolic activities, (3) hyperimmunity response, and (4) gene transfer. Therefore, additional tests are needed as follows: (1) Determination of antibiotic resistance patterns; (2) assessment of certain metabolic activities
such as \(\text{d}-\text{lactate}\) production, bile acid deconjugation; (3) assessment of side-effects during human studies; (4) epidemiological surveillance of adverse incidents in consumer at post-market; (5) if the strain under evaluation belongs to a species that is a known mammalian toxin producer, it must be tested for toxin; and (6) if the strain under evaluation belongs to a species with known hemolytic potential, determination of hemolytic activity is required.

Approved probiotic microorganisms are listed below (Table 32.10). Additional microbial species can be checked from Bulletin of the International Dairy Federation 377/2002.

If microorganisms are not mentioned in the list and would like to claim as probiotics, its probiotic property would need to be assessed as mentioned above. *Enterococcus* having virulence property is not suggested to be used for human probiotics unless it has been proven that it is not antibiotic resistant, has no vancomycin resistant gene transfer system, no virulent property, and would not cause infection.

### 32.10.3.4 Efficient Assessment of Probiotics to Health

After approval of probiotics for safety, scientific substantiation of probiotic efficiency in humans is needed. A well-designed human intervention study or a well-designed randomized controlled clinical trial is needed to be performed by at least two institutes. The design of studies should include the following parameters: (1) type of food and serving size, (2) quantity of probiotic microorganism, (3) utilization time to gain the benefit, and (4) scientific evidence. These scientific evidences should include (Aggett et al. 2005; Richardson 2005a,b, 2007)

1. Food or food composition claimed must be clearly identified.
2. Study groups should match as nearly as possible the target group with appropriate control.
3. An adequate duration of exposure and follow up to demonstrate the intended effect. Thus, there should be a suitable period of exposure to the food or food component (period of intake) and the duration of observation is long enough for the expected effect to occur, and if necessary, to show that the benefit is sustained.
4. The substantiation of claims should include characterization of the study groups’ background diet and adjust not only for diet, but also for lifestyle factors that might affect the outcome of the study.

5. The amount of food or food component that will be tested should match its intended use and the way and frequency with which it will be eaten. Where dose–response studies are performed, the range of dose must include the amount of food or food component expected to be consumed.

6. In any study of diet and health, it is necessary to know the actual dietary intake of the subjects and to confirm that they have taken the food or food component in question in the right amount at the right time and over the specified period.

7. Studies providing evidence for a claimed effect of a food should indicate the statistical criteria that were used in the design of human intervention trials.

8. In the case that the true endpoint of a claimed benefit cannot be measured directly due to several possible reasons: for example, (a) a long duration between the introduction of the intervention and the desired outcome, (b) ethical concern, and (c) biochemical evaluation in a large-scale study demanding expertise and resources which might be unpractical and expensive. Then, more easily measured markers may be used.

9. Markers mentioned in item 8 should be (a) biologically valid and (b) methodologically valid.

10. The target variables should change in a statistically significant way and the change should be biologically meaningful for the target group and consistent with the claim to be supported.

11. A claim should be scientifically substantiated by taking into account the total available data and by weighing of the evidences of (1) consistency of results across the various categories of evidences and methodologies; (2) valid dietary methods; (3) randomized sampling; (4) a dose–response relationship between intakes of food or food components and the health effects, if relevant; and (5) biological plausibility.

32.10.3.5 Appropriate Labeling

To clarify the identity of a probiotic, labeling of products should follow the regulation of the Ministry of Public Health Thailand relating to labeling issue including additional information as below:

1. Genus, species, and strain of probiotic used should not be misleading with respect to its functionality.

2. Lowest cell survival concentration of $10^6$ CFU/g needs to be verified at the end of shelf-life.

3. Suggestion of enough quantity and consumption time affecting health claims should be defined.

4. Health claims are required to pronounce.

5. Suitable storage conditions are required to pronounce.

6. Contact address to provide more information to consumers is required to pronounce.

The scope of health claims could be (a) nutrient function claim, (b) special beneficial effect or improvement of function, for example, calcium absorption, and (c) reduction of disease risk claim.

The health claim labeling should consider the following concerns: (1) It should be safe; (2) its health claims should be from food or food components directly, and should not be because of co-consumption with other components; (3) nutrition labeling should be performed according to the guidelines of the Ministry of Public Health; (4) health claims should be from food component
not a food product except when there is some scientific evidence; and (5) health claims of food component should be written in Thai. It is allowed to use other language with the same meaning and letter size on the same label, and (6) it is not allowed to use the word “therapy, disease protection” for labeling.

Finally any health claims need to be considered and allowed by FDA, Ministry of Public Health Thailand.

Acknowledgments

The authors thank the following for the assistance in the preparation of this chapter: Dr. Nandini Kumar, Retd. Senior Deputy Director General, Indian Council of Medical Research; Professor Wang June Kim, Department of Food Science and Technology, Dongguk University, Seoul, Korea; Ms. Lilim Lee and Lynn Whui Chow, Health Supplements Unit, Complementary Health Products Branch, Health Products Regulation Group, Health Sciences Authority of Singapore; and Thai Food and Drug Administration, Ministry of Public Health, Thailand.

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Chapter 33

Regulation of Probiotic and Probiotic Health Claims in South America

Célia Lucia De Luces Fortes Ferreira and Marcelo Bonnet

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33.1 Introduction

As observed worldwide, the aged population in South America (SA) has increased, and likewise the incidence of chronic and degenerative diseases. Functional foods or foods with special health use are an alternative to delay and/or to help prevent some of those diseases. Arguably, the consumer in SA has seen an increase in the offer of such foods.
Prebiotic ingredients, probiotic microorganisms, and probiotic food products are among the most studied foods and/or bioactive components, comprising the majority of the food products being regulated and registered as functional (Hawkes 2004). More than a decade ago, Brazil was the only country in SA carrying legislation on functional foods. Today, some other countries from the MERCOSUL or MERCOSUR (Southern Common Market) are in the process of implementing their regulations based on the experience of Brazil. Because functional food products are similar to the standard products available in the market, suitable labeling and health claims are critical to differentiate them. In general, health claims are an increasingly important issue to the consumer, to the public health programs, and to the food industry. It allows the industry to develop and to provide differentiated products to the market, allows the consumer to be informed about potential benefits of a given product, and helps authorities to strategically manage national programs to control chronic and degenerative diseases. Accordingly, nutrition labeling and health claims can aid and support the consumer decision to purchase foods associated with specific health benefits, along with positive impacts for national public health systems. As such, they should require regulatory interventions, according to the current Brazilian experience. In this chapter, guidelines for nutrition, probiotic, probiotic health claims legislation, and regulatory aspects enforced in different countries in SA are reviewed, with special focus to policies as implemented in Brazil, the first country to regulate such issues in the region.

33.2 Nutrition Transition and Functional Foods

In the last four decades, the nutritional status of the populations all over the world has been affected by the increase in industrialization, urbanization, and by market globalization. This worldwide trend is also true in SA, where the shift of the populations to urban areas resulted in a decline of under-nutrition in such areas, but at the same time has caused increased adoption of inadequate dietary patterns and decreased physical activity (Lajolo 2002). In the public health segment, diseases related to either deficiency or excess of food are part of what is known as the “nutrition transition.” The main factors associated with the increase of chronic diseases as seen by Popkin (2010) are demography (from high mortality and high fertility, to low mortality and high longevity), epidemiology (from high to low incidence of infectious diseases), and nutrition (from high to low prevalence of undernutrition), combined with decreased physical activity and increased consumption of high-calorie foods, often containing high levels of saturated fats.

Illnesses resulting from nutrition transition include cardiovascular diseases, obesity, diabetes, hypertension, osteoporosis, and cancer, all belonging to the class of chronic diseases. Chronic diseases are promoted by decreased consumption of fruits and vegetables, complex carbohydrates and, therefore, micronutrients—patterns usually associated with a sedentary lifestyle. These diseases are responsible for premature mortality, for increases in disabilities, as well as for the rising burden on public health systems in several countries’ budget. However, a number of chronic diseases can be prevented or at least postponed, and one of the strategies to decrease such diseases is the implementation of consumer education programs. As such, appropriate nutrition labeling and credible, validated health claims represent a key step toward this educational process. In this respect, probiotic products and their claims could be seen as a potential addition to the toolbox of interventions to prevent chronic diseases.

33.3 Probiotic and Probiotic Health Claims: Defining the Terms

A terminology work has recently been published covering the main terms involved in functional foods, probiotic and health claims issues, among others (Magalhães et al. 2011). This work was triggered by
the difficulty to comprehensively define those terms in different regions, countries, and communities, combined with the necessity for developing a global understanding for future developments in the area of functional foods and probiotics products with therapeutic uses and health claims.

Education, information carried by advertisements, and the labels are usually described as the main variables involved in the consumer’s decision for purchasing a product (Hawkes 2004). Moreover, the label usually identifies and informs the consumer about the specific characteristics of the product such as its origin, composition, nutrient contents and, more recently, nutritional and health benefits. When they do appear on the label, the latter are considered as “nutrition claim,” “health claim,” and or “functional claim.” Food labels are increasingly being used by consumers to guide them about the food they intend to purchase or to eat (Coutinho and Recine 2007). In the Americas, as in other regions of the world, the Codex Alimentarius, created by the World Health Organization (WHO) and the Food and Agriculture Organization (FAO) of the United Nations, is the global reference for food quality and identification standards. Since 2004, the Codex Alimentarius has set forth guidelines for the use of nutrition and health claims, and states that the health claims must be consistent with national health policies. Moreover, each country should decide whether a claim should be permitted, even if it conforms to Codex principles, “because adoption of some wide-ranging restriction policies might lead to trade barrier” (Hawkes 2004).

According to the Codex definition, a claim is “any representation which states, suggests or implies that a food has particular characteristics relating to its origin, nutritional properties, nature, production, processing, composition or any other quality” (Codex Alimentarius 1991). A health claim is any representation that states, suggests, or implies that a relationship exists between a food, or a constituent of that food, and health (Codex Alimentarius 2009). The same term has been adopted by the European Community (EC) as “any message or representation, which is not mandatory under Community or national legislation, including pictorial, graphic or symbolic representation, in any form, which states, suggests or implies that a food has particular characteristics” (EC 2006; Magalhães et al. 2011).

A food with a health claim is defined as “food similar in appearance to a conventional food (beverage, food matrix), consumed as part of the usual diet which contains biologically active components with demonstrated physiological benefits and offers the potential of reducing the risk of chronic diseases beyond basic nutritional functions when carrying a health claim” (EC 2006; FAO/FQSS 2007; Magalhães et al. 2011).

The term functional food has the same definition as that previously indicated for foods associated with a health claim (FAO/FQSS 2007). Functional foods carry one or more active compounds or components that impart reasonably demonstrated beneficial properties to the food, for example, flavonoid, carotenoid, terpene, fiber (prebiotics), as well as specific microorganisms (probiotics), to mention a few.

A probiotic product contains probiotic microorganisms intended to be consumed as food, feed, or nonfood, with or without medical supervision, whereas a probiotic food product is a food product containing probiotic microorganisms intended for human consumption. Furthermore, according to the recently published terminology compilation mentioned earlier (Magalhães et al. 2011), a probiotic claim is “any representation that states, suggests or implies that a probiotic food has particular characteristics relating to its origin, nutritional properties and health.”

According to the Codex Alimentarius (1991), there are two types of food claims—nutrient claims and health claims. The latter can be further divided into nutrient function claim, reduction of diseases risk claim, and other function claim.

A nutrient function claim describes the physiological role of the nutrient in growth, development, and normal functions in the body. The definition of this claim had been recently moved
from the nutrient claim section to the health claim section of the Codex guidelines (Magalhães et al. 2011).

Reduction of disease risk claim is “any health claim that states, suggests or implies that the consumption of a food category, a food or one of the constituents significantly reduces a risk factor in the development of a human disease” (EC 2006). Given that diseases can be associated with multiple risk factors, altering one of these risk factors may or may not have a beneficial effect. For this reason, an important aspect of the interpretation of this term is that the use of labels containing a risk reduction claim must not lead consumers to interpret them as prevention claims (Codex Alimentarius 2009).

Other function claim indicates specific benefits derived from the consumption of foods or their constituents, in the context of the total diet on normal functions or biological activities of the body. Such claim refers to a positive contribution to health or to the improvement of a specific function, or even to preserving the health status or improving it. Other function claim used to be termed “enhanced function claim,” and does not include nutrient function claims (Magalhães et al. 2011).

The term referring to “food health claim” as adopted in Brazil by the National Health Surveillance Agency (Agência Nacional de Vigilância Sanitária, ANVISA) is “alegação de propriedade de saúde,” defined as a claim that states, suggests, or implies the existence of a connection between the food or ingredient with disease mitigation or condition related to health. Health claims that mention cure or prevention of diseases are not allowed in Brazil (Brasil 1999c).

Another possible food claim under current Brazilian regulation is the “functional food claim,” which is related to the metabolic or physiological role that the nutrient or non-nutrient plays in the growth, development, maintenance, and other normal functions of the human organism (Brasil 1999c). An example of such an attribute on the label could be: “foods fibers help bowels work well. Intake must be associated with a balanced diet and healthy habits” (Brasil 2006).

Thus, the different food health-related claims in Brazil fall into two categories only—functional claim or health claim.

Nutrition and health labels are voluntary, but become mandatory whenever a claim is made. Producers understand the advantages of carrying nutrition and health claims for their products, and use them as a marketing instrument. Because consumers are increasingly aware of such claims, precautions should be taken that these messages do not confuse or mislead the target buyer (Hawkes 2006).

### 33.4 Health Claims: Global Concerns

The presence of a health claim on the label of a product could stimulate the choice and consumption of healthy foods, but this practice could lead to overconsumption of specific products and nutrients (Hawkes 2004). For this reason, health claim legislation should be made in the context of the whole diet matrix, and not be focused on a specific food nutrient. Furthermore, the alleged benefit should be based on reasonable consumption.

Also of concern is that a health claim for a product might indicate that it is “better” than others in the same category and, because of this, such claims should not be allowed in products that should be consumed in moderation. On the other hand, too stringent label regulations for health claims could be perceived as a form of commercial barrier, as advised by the Codex Alimentarius, in its critical effort to constructively guide national governments to establish legislation that promotes quality, safe food products, and fair practices in food trade. These issues are to be taken into account by the regulatory agencies concerned with health claims for foods (Hawkes 2004).
Probiotic and Health Claims in South America

MERCOSUL is a Regional Trade Agreement which aims at promoting full trade and fluid movement of goods, people, and currency between the member countries. Established in 1991 by the Treaty of Asunción, it was later amended and updated by the 1994 Treaty of Ouro Preto. The official languages are Portuguese and Spanish. Full members of MERCOSUL are Argentina, Brazil, Paraguay, and Uruguay. Venezuela has applied for membership in 2006, but its petition is still pending. Currently, Bolivia, Ecuador, Peru, and Chile have associate membership status, whereas Mexico holds observer status. The only non-South American trade partner country is Israel (MERCOSUL 2010). Currently, no specific regulations on probiotic or probiotic health claims exist in countries of SA other than Brazil and, when necessary, the regulation already established in Brazil is applied (Hawkes 2004; Desjardins 2006). Thus, the definitions, decrees, and laws regulating these issues in Brazil will be more deeply discussed.

Probiotic and Probiotic Health Claims in Brazil

Functional foods in Brazil make up a large portion of the Brazilian health and wellness market, and combined with fortified foods, showed an 8% growth in 2007, and sales in excess of US$4 billion. Dairy products predominate, accounting for 59% of all functional foods sales, which hold a perspective growth of 5% between 2007 and 2012 (Nutra Ingredients 2009).

Brazil was the first country in SA to legislate functional foods and their claims. Probiotic and their health claims are regulated in Brazil by the ANVISA, or National Health Surveillance Agency, which has been created by Federal Law number 9.782 on January 26, 1999 (Brasil 1999e). ANVISA is linked to the Ministry of Health, yet has an independent administrative Collegiate Directory. The main function of ANVISA is to promote health protection of the population through the control of the sanitary production and commercialization of food products and services, including the environment, processes, ingredients, and related technologies. Sharing responsibilities with the Ministry of Agriculture, Livestock and Food Supply, and the Ministry of Environmental Affairs, ANVISA has representatives in ports, airports, and frontiers, and aids the Ministry of Foreign Affairs Foreign Ministry in addressing international issues in the area of sanitary surveillance. ANVISA also establishes norms and legislations, and enforces some policies related to product registration.

The roots of the current functional foods and health legislation in Brazil were started with Act 1549, from October 17, 1997 (Brasil 1997), describing different food additives and supplements, including the definition of foods for special uses. However, the act did not specify the ruling of labeling criteria for such products, and this lack of guidelines and misinterpretation of the Act resulted in a large amount of additives and supplements being commercialized “as food” without approved labeling and claims. Another problem was the increasing number of inquiries to the Health Ministry, made by companies willing to commercialize functional foods. These facts required the development of more specific regulations and, as a result, resolutions 16, 17, 18, and 19 came into force in 1999.

ANVISA has the competence, among others, to register functional products, establish norms, and reinforce policies and actions under the scope of sanitary surveillance. A group of specialists acting as a Consulting Technical Scientific Advisory Commission on Functional Foods and New Foods (CTCAF) was established (Brasil 1999f). This group routinely meets to discuss issues related to the registration and approval of functional and new foods, as well as to debate their functional and health claims.
Resolutions 16, 17, 18, and 19 established the basis for the understanding of functional foods, functional and health claims, and the guidelines for their registration (Brasil 1999a–d). Yet only in 2002, a resolution came into force approving probiotic functional and health claims (Brasil 2002). Brief information on the contents of such resolutions follows.

Resolution 16, of April 30, 1999 (Brasil 1999a) deals with the TECHNICAL REGULATION ON PROCEDURES FOR REGULATION OF FOODS AND/OR NEW INGREDIENTS. The regulation is intended to be used for the registration of new foods, and/or ingredients for human consumption without a record of consumption in Brazil, or foods containing added substances already consumed as such, or the consumption of products containing substances at much higher levels than those normally present in the foods that make up a regular, average diet. Additives and food manufacturing adjuvants are excluded from this regulation.

For the purposes of this regulation, the interested party must present the following documents to the competent authority: (1) Technical Scientific Report containing the following information: name of the product; purpose of use; recommended intake indicated by the manufacturer; scientific description of the ingredients of the product, according to species of botanical, animal, or mineral origin, when appropriate; chemical composition and molecular characterization, when appropriate; formulation of the product; description of the analytical method for evaluation of the food or ingredient that is the object of the petition; applicable scientific evidences, as appropriate, to prove the safety of intended use; nutritional and/or physiological and/or toxicological assays; biochemical assays; epidemiological studies; clinical assays; evidence of traditional use, observed in the population, without damage to health; broad evidences from the scientific literature, international health organizations, and internationally recognized legislation on the characteristics of the food or ingredients; (2) foods intended to be commercialized in the form of capsules, pills, or other pharmaceutical formulas, and that do not present scientifically proven functional or health claims, must display the following information on the label: “The Ministry of Health warns: there is no proven scientific evidence that this food prevents, treats or cures diseases”; (3) any information, including functional or health claims for a food or ingredient, as advertised by the media, cannot be different in meaning from the information approved for display on its label; (4) technical scientific evaluation report on the risk and demonstration of safety, to be evaluated by CTCAF, instituted by ANVISA. The Brazilian Health Surveillance Agency shall revise this regulation within a minimum of 2 years after it enters into force.

Resolution 17, of April 30, 1999 (Brasil 1999b): TECHNICAL REGULATION ESTABLISHING THE BASIC GUIDELINES FOR EVALUATION OF RISK AND SAFETY OF FOODS. This is applied to foods and ingredients for human consumption. Some terms are defined and protocols to prove safety are indicated. Safety proof should be conducted based on information of purpose, and conditions of use, of the food or ingredient. The risk evaluation is based on one or more scientific evidences, according to the circumstances. Among the necessary information are: (1) chemical composition and molecular characterization, when appropriate, and/or formulation of the product; (2) nutritional and/or physiological and/or toxicological assays in experimentation animals; (3) epidemiological studies; (4) clinical assays; (5) general evidence from scientific literature, international health organizations, and internationally recognized legislation on the characteristics of the food or ingredient; and (6) evidence of traditional use, observed in the population, without damage to human health. Unforeseen or special situations are managed on a case-by-case basis by the CTCAF.

Resolution 18, of April 30, 1999 (Brasil 1999c): This TECHNICAL REGULATION establishes the GUIDELINES FOR ANALYSIS AND PROOF OF FUNCTIONAL AND/OR
HEALTH CLAIMS ON FOOD LABELS. It defines functional and health claims and establishes the guidelines for using such claims. For nutrients with functions fully recognized by the scientific community, the demonstration or analysis of effectiveness shall not be necessary for a functional claim on the label. However, when a new function is claimed, there is a need for scientific evidence of the functional and/or health claim and/or safe use, according to the Basic Guidelines for Evaluation of Risk and Safety of Foods. Health claims that mention cure or prevention of diseases are not allowed.

Resolution 19, of April 30, 1999 (Brasil 1999d): TECHNICAL REGULATION ON PROCEDURES FOR REGISTRATION OF FOODS WITH FUNCTIONAL AND/OR HEALTH CLAIMS ON THEIR LABELS. It applies to health and functional claims, defining and establishing guidelines for their use. The resolution indicates that the proof of functional and/or health claims for foods and/or ingredients must be conducted based on: (1) estimated or recommended intake provided by the manufacturer; (2) purpose; (3) conditions of use and nutritional value; and (4) scientific evidence. Unforeseen situations are managed by the CTCAF.

Resolution RDC 02/2002, of January 7, 2002 (Brasil 2002): TECHNICAL REGULATION OF ISOLATED BIOACTIVE SUBSTANCES AND PROBIOTICS WITH FUNCTIONAL AND HEALTH PROPERTIES CLAIMS. Among the items that motivated this resolution are: (1) the need to constantly improve the actions on prevention and sanitary control in the food sector to enhance the health of the population; (2) the potential health benefits from nutrients and bioactive substances from foods; (3) the need to provide means to evaluate safe uses of bioactive substances; (4) the possibility that the ingestion of such substances in a short and/or long run could result in adverse effects to the consumer; and (5) the possibility of occurrence of interactions between bioactive substances and nutrients, or non-nutrients, in the body, with undesirable health effects, in a short or long run.

The aim of the resolution was to standardize the guidelines to be adopted to evaluate the safety, to register and to commercialize isolated, bioactive substances and probiotics with functional and health claim properties. Some terms are defined such as raw material, nutrient, probiotic, bioactive substance, and isolate. Among other directives, it distinguishes a functional food from an active compound marketed in nonfood formats such as capsules/pills, and others that fall under pharmaceutical regulations. The classes of products under this legislation are carotenoids, phytosterols, flavonoids, phospholipids, organosulfur compounds, polyphenols, fibers, and probiotics. It indicates requirements for product labeling, registration, and advertising. With regard to advertising, any information about the claims of the product relayed in any communication form shall not differ from that already approved for labeling. It is worth noting that the approval covered by this resolution relates to the food product containing the isolated bioactive compound or probiotic, and not to the isolated compound or probiotic itself.

Recent consultation on the approved substances and claims list indicates as probiotic microorganisms: *Lactobacillus acidophilus*, *Lactobacillus casei* Shirota, *Lactobacillus casei* var. *rhamnosus*, *Lactobacillus casei* var. *defensis*, *Lactobacillus delbrueckii* ssp. *bulgaricus*, *Lactococcus lactis* ssp. *lactis*, *Bifidobacterium bifidum*, *Bifidobacterium animalis* (including the subspecies *B. lactis*), *Bifidobacterium longum*, and *Enterococcus faecium* (Brasil 2010). As exemplified, a probiotic claim should read: “The microorganism X contributes to the intestinal flora balance. Consumption should be associated to a balanced diet and healthy life habits.” Among its requirements, it indicates that the minimum quantity of viable probiotic cells should be in the range of $10^8–10^9$ colony forming units (CFU) via the daily recommended dose of the product ready to be consumed, according to manufacturer’s indication. Lower values could be accepted under proof of efficacy provided by the proponent. Documentation required to prove efficacy include: (1) report of the
product with proof of minimum numbers of viable microorganisms at the end of shelf life, (2) tests indicating that the microorganism resists gastric acidity and bile salts, and (3) the amount of probiotic organisms as CFU present in the recommended daily consumption of the product ready to eat should be indicated on the label, in the vicinity of the claim (Brasil 2010). Claims such as “increased antibodies” and “strengthens natural defenses against daily aggression and stress” are not allowed (Hawkes 2006).

An aspect of concern is that although there is a guideline for health claims, registration of such claims depends on documented information, often from international sources, because human studies in Brazil to substantiate such claims are rare to nonexistent (Hawkes 2006). A noteworthy characteristic of the approved functional and/or health claim in Brazil, however, is that they are continuously under official surveillance and scrutiny.

As a country member of MERCOSUL, Brazil must comply with the harmonized resolutions adopted by the Common Market Group (CMG). Before approval, the harmonized draft resolutions are submitted for public consultation. The adopted resolutions by MERCOSUL should be internalized and incorporated into the national legislation of member countries through the relevant bodies of each of those countries (Coutinho and Recine 2007).

33.5.2 Health Claims in Other Countries of South America

33.5.2.1 Argentina

Before 2006, the health and nutrition claims in Argentina were regulated and registered as modified foods by the Ministry of Public Health. The criteria were those set forth by the Codex Alimentarius, the United States Food and Drug Administration (FDA), and the Argentinean Bromatologic National Regulation 315/994, as well as Internal Technical Regulations of MERCOSUL (Desjardins 2006).

More recently, on March 2009, the addition of health claim properties as Art 236 to Chapter V: Labeling of the Argentine Food Codex (CAA) was requested by the Coordinadoria de las Industrias de Productos Alimenticios. The increase in the demand for functional foods, the increase in research data and information indicating the beneficial effect of such foods, the new trend in consumers that are more oriented to a healthier diet, and the importance to have credible, regulated claim on food labels were the basis for such petition. Thus, during the Meeting 82 of the National Commission of Foods (Comisión Nacional de Alimentos, CONAL), held on June 29, 2009, a working group was formed to evaluate the petitioned document. This commission is currently responsible for assisting food demands within the National System of Food Control (Sistema Nacional de Control de Alimentos, SNCA). The members represent different levels of government, among them, the Ministry of Agriculture. Their responsibilities should include the surveillance and enforcement of the CAA throughout the country, and the provision of the necessary adjustments to the international norms and agreements, including those decided in the realm of MERCOSUL (Ministerio de Agricultura, Ganadería y Pesca 2010).

33.5.2.2 Bolivia

As a Country member of MERCOSUL, Bolivia must comply with the harmonized resolutions adopted by the CMG. Resolutions and directives adopted by MERCOSUL should be incorporated into national legislation through the relevant bodies of each country (Coutinho and Recine 2007).
33.5.2.3 Chile

In 2003, the term “Functional Foods” was yet not recognized by the Chilean Legislation, and the accepted claims were those approved by the US FDA (Araya and Lutz 2003). The term health claim is not recognized by the Ministry of Health in Chile (Araya et al. 1996). Instead, a “claim of health properties” in foods is adopted, which is similar to the terminology accepted in Brazil, and to that indicated by the Codex Alimentarius (Coutinho and Recine 2007).

33.5.2.4 Mexico

Mexico is a country state holding observer status in relation to MERCOSUL. In 2006, functional claims were not recognized and regulatory issues were still under construction (Desjardins 2006).

33.5.2.5 Paraguay

As a country member of MERCOSUL, Paraguay shall comply with the harmonized resolutions adopted by the CMG (Coutinho and Recine 2007).

33.5.2.6 Uruguay

The Ministry of Public Health is responsible for defining and regulating functional foods through a multisectorial commission (Scoseria 2004). However, after 2006, MERCOSUL members complied with the harmonized resolutions adopted by the CMG (Coutinho and Recine 2007).

33.6 Conclusion

Probiotic and probiotic health claims are currently important issues in SA, where the most important trading block is the MERCOSUL. Functional foods are in high demand in the region, and constitute one of the most rapidly growing sectors of its food industry. This trend is also observed in other regions of the globe, and the commercialization of such food products arguably requires special legislation. Probiotic microorganisms and probiotic food products comprise the majority of the foods being regulated and registered as functional and dairy products lead this trend. Many chronic diseases may be modulated by the educated consumption of such products, which makes them important tools to help public health programs attain their goals. However, as these products appear similar to conventional products, often displayed close to each other, reliable, appropriate labeling should help consumers in their educated decision-making process. Although probiotic and probiotic health claims should be specially legislated, few countries in the world possess official regulations and directives on functional foods. Notably, Brazil was the first country in SA to set forth and enforce a consistent, science-based legislation on functional foods, as well as directives on health and functional claims for food products. The other member countries of MERCOSUL have used the Brazilian Regulation to help format their own regulations, which are still under construction in varying degrees. In the meantime, the country members of MERCOSUL must comply with the harmonized resolutions adopted by the CMG. Before their approval, harmonized draft resolutions are submitted to public consultation, and the adopted resolutions by MERCOSUL should be incorporated into national legislation through the relevant bodies and competent authorities of each country.
References


While lactic acid–producing fermentation has long been used to improve the storability, palatability, and nutritive value of perishable foods, only recently have we begun to understand just why it works. Since the publication of the third edition of Lactic Acid Bacteria: Microbiological and Functional Aspects, substantial progress has been made in a number of areas of research. Completely updated, the Fourth Edition covers all the basic and applied aspects of lactic acid bacteria and bifidobacteria, from the gastrointestinal tract to the supermarket shelf.

Topics discussed in the new edition include:

- Revised taxonomy due to improved insights in genetics and new molecular biological techniques
- New discoveries related to the mechanisms of lactic acid bacterial metabolism and function
- An improved mechanistic understanding of probiotic functioning
- Applications in food and feed preparation
- Health properties of lactic acid bacteria
- The regulatory framework related to safety and efficacy

Maintaining the accessible style that made previous editions so popular, this book is ideal as an introduction to the field and as a handbook for microbiologists, food scientists, nutritionists, clinicians, and regulatory experts.