YOGURT in Health and Disease Prevention

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Nagendra P. Shah

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Preface

Yogurt, a traditional fermented dairy product, has shown a remarkable resurgence and growth in the past few decades. This increased worldwide popularity of yogurt can largely be attributed to the health benefits associated with its consumption. Additionally, product innovations, leading to availability of diverse flavors and types of yogurt, have contributed significantly to the enhanced consumption. The per capita consumption of yogurt has been steadily increasing in most countries around the world. Over the past five years, most of the world’s major economies have reported more than 10% increase in purchase (or sales volume). In the United States, retail sales of yogurt are expected to be at $9 billion in 2017. For the industry, yogurt is advantageous as it provides a convenient platform for increasing the consumption of dairy produce by adding components to either increase palatability and/or appearance. There is also an increasing awareness that yogurt is an important source of nutrients and nutraceuticals. The versatile nature of yogurt also means that novel components can be added that have the potential to confer additional health benefits. The modifications or additions to yogurt include altering the protein-to-carbohydrate ratios; making protein-rich snacks; adding $n$-3 fatty acids, plant stanols and sterols; making yogurt with oats; and adding olive-derived products or vegetable extracts.

Moreover, there are pre-, pro-, and synbiotic yogurts with wide-ranging health-conferring benefits. In general, this book presents a contemporary update and a unique approach to encompass yogurt production and composition; yogurt-like beverages made with cereals or carrot juice; microbiology of yogurt and bio-yogurts containing probiotics and prebiotics; yogurts with added omega-3 polyunsaturated fatty acids, plant sterols and stanols, olive-derived products, beta-casomorphins, estrogenic compounds; and the roles of yogurt in cardiovascular health, nutrition and health of children and adolescents, bone health and on slowing the progression of HIV. The book also presents yogurts and yogurt-type products known around the world, as well as regulatory aspects.

In some countries there are specific types of yogurts that may be in the semisolid or beverage form. However, little is known about these international yogurts from countries such as Ghana, Iran, Turkey, and Bulgaria. Potentially, they may offer hitherto unrecognized health-related properties and opportunities for extracting health-related components.

Finding all of the aforementioned information in a single source has hitherto been a problem as there is such a wide range of scientific material and information related to yogurt. All this information has been compiled in this book, and it is designed to augment related books in the existing market.

This book is designed for nutritionists and dietitians, food industry professionals, public health scientists, doctors, epidemiologists, health care professionals of various disciplines, policy makers, and marketing and economic strategists. It is designed for teachers and lecturers, undergraduate and graduate students, researchers, and professors.

I assembled 30 authors from the United States, Canada, Australia, United Kingdom, Ireland, Hong Kong, India, Turkey, Iran, Italy, Finland, Spain, Bulgaria, Greece, Brazil, Ghana, Thailand, Vietnam, the Netherlands, Denmark, and Switzerland to write the chapters. These contributors are authors of international and national standing, leaders and trendsetters in the field, and represent diverse expertise from academia, food and dairy industry, and research institutions to ensure up-to-date information and scientific accuracy.
The book includes five major sections: (1) Yogurt from lab to table (Chapters 1–6); (2) Yogurt additives and reformulations (Chapters 7–14); (3) Yogurts around the world (Chapters 15–20); (4) Important micronutrient and biotic components of yogurts (Chapters 21–26); and (5) Yogurt and human health (Chapters 27–30).

Chapter 1 gives an overview of yogurts, historical background, their popularity, and global trade. Chapters 2 and 3 deal with an overview of yogurt production and composition and use of stabilizers, colorants, and exopolysaccharides. Chapter 4 discusses microbiology of yogurt and bio-yogurts containing probiotics and prebiotics. In addition to yogurt, other fermented dairy products including kefir and koumiss are discussed in Chapter 5. Chapter 6 covers regulatory aspects of yogurt.

Chapter 7 deals with yogurt with added omega-3 polyunsaturated fatty acids, and Chapter 8 is devoted to yogurt made with plant sterols and stanols. Chapter 9 describes potential applications of prebiotics to yogurt and their impact on health. Chapter 10 covers yogurt-like beverages made with cereals. Chapter 11 discusses functional yogurt enriched with olive-derived products. Chapter 12 is dedicated to composition, microbiology, and sensory acceptability of carrot juice yogurt. Chapter 13 deals with effects on fecal microbiota on probiotic yogurt intake. Chapter 14 describes synbiotic yogurts and their influence on the elderly.

Chapters 15–20 focus on international aspects of yogurt especially from nations such as Ghana, Iran, Turkey, Bulgaria, Brazil, and India. These chapters discuss manufacturing practices and popularity of nunu, a West African fermented yogurt-like milk product, popularity of traditional yogurt in Iran, rheology and functionality of ayran, a yogurt drink popular in Turkey; the popularity of katak, a Bulgarian yogurt-like product; popularity and consumption of a Brazilian yogurt-like product; and the manufacturing practices and consumption of dahi, an Indian naturally fermented yogurt.

Chapter 21 discusses the role of biotic beta-casomorphins in yogurt. Chapter 22 is devoted to the role of lactose in yogurt. Chapter 23 covers the role of bacteria in yogurt and strain-dependent effects on gut health. Chapter 24 contains information on bioactive peptides in yogurt and their role in human health. Chapter 25 discusses the role of metabolomics as emerging strategy for investigation of yogurt components. Chapter 26 covers the role of estrogenic compounds in yogurt.

Chapters 27–30 highlight the role of dairy and yogurt on cardiovascular health, nutrition and health of children and adolescents, bone health, and on reducing the progression of HIV.

I have significant experience in the science of yogurt, yogurt manufacture and its applications, biotic and bio-yogurt, and probiotics, prebiotics, and synbiotics, and their role in human health. I have received several prestigious awards and accolades for outstanding contributions to the field. It is hoped that Yogurt in Health and Disease Prevention will appeal to nutritionists and dietitians, food industry professionals, medical professionals, public health scientists, health care professionals, teachers and lecturers, undergraduate and graduate students, researchers, and professors.

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YOGURT PRODUCTION FROM LAB TO TABLE
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YOGURT: HISTORICAL BACKGROUND, HEALTH BENEFITS, AND GLOBAL TRADE

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1.1 INTRODUCTION

The word *fermentation* was derived from a Latin word *fervere* and was later defined by Louis Pasteur as “life without air.” It is a metabolic process that derives energy from the organic compounds without the involvement of any exogenous agents. Yogurt, a fermented milk product, is defined as “a product resulting from milk by fermentation with a mixed starter culture consisting of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*.” Although *S. thermophilus* (ST) and *L. delbrueckii* ssp. *bulgaricus* (LB) are the common yogurt starter bacteria, over the past few decades several other adjunct bacteria have been used in addition to these for production of yogurt. Some of the other commonly used bacteria for yogurt production are *Lactobacillus helveticus*, *Lactobacillus casei*, and *Lactobacillus jugurti* and several species of *Bifidobacterium*. For instance, in Australia, yogurt can also be prepared with ABT (*acidophilus*, *Bifidobacterium*, and *thermophilus*) starter culture, which uses the bacteria *S. thermophilus* and *Lactobacillus acidophilus*, and *Bifidobacterium* spp., with *S. thermophilus* being the primary fermenting bacterium in this ABT starter culture group.

Commercial production of yogurt involves heat treatment of milk containing extra milk solids-not-fat and other additives at 85°C for 30 min, cooling to 43°C and inoculation with 2% starter culture (*S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus*). The inoculated yogurt base is allowed to ferment and coagulate by incubation at 42°C for about 4 h until 0.9% acidity is reached. The fermented yogurt base is cooled to 4°C to arrest further growth of the culture and further acid development. This base forms the starting material for production of more popular fruit-flavored yogurt. The United States (US) federal standards of identity for yogurt were first established in 1981. The US standards (FDA, 2011) mandate the production of yogurt with characterizing culture consisting of *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus*. Other probiotic organisms are permitted as additional cultures. Under Canadian law, a product must contain the two characterizing bacteria (*S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus*) to use the name “yogurt.” With the advent of probiotic organisms, their supplementation in yogurt also became popular. Probiotic bacteria, such as *L. acidophilus* and *Bifidobacterium* spp., are commonly included in probiotic yogurt to enhance the health benefits of yogurt. Recent advances also include addition of certain high exopolysaccharide-producing bacteria for textural enhancements of yogurt (Prasanna et al., 2013).
1.2 YOGURT: A HISTORICAL PERSPECTIVE

Historically, fermentation was used by humans for preservation of milk. Although there are no records to trace back the origin of fermented milk products, it is thought to have originated in the Middle East area even before the Phoenician era. In Egypt, the consumption of traditional fermented milk beverages such as laban rayeb and laban khad dates back to around 7000 BC. The Vedas (Indo-Aryan treatises) also mention dadhi (a fermented milk product resembling modern-day yogurt) dating back to c.5000 years BC. Dadhi was touted to possess therapeutic properties. Dadhi or dahi is still a crucial component of the South Asian diet. It is produced in most Indian households and consumed daily. The word yogurt is believed to have been first used by the Turks in the 8th century, which appeared as yoghurut. It is thus assumed that the Turkish nomads in Asia made yogurt. Another legend, however, states that yogurt was first prepared or invented by the Balkan people. Sour milk, prokish, was prepared from sheep’s milk by the peasants of Thrace. Persian societies believe that Abraham (often also called Ibrahim) lived longer due to regular yogurt consumption. South Asian regions (India, Pakistan, Nepal, and Bangladesh), as well as southwest Asia regions (Iran, Iraq, Balkans, Turkey, Syria) are among the largest producers and consumers of fermented milk products (including yogurt). It is believed that the invasion of Mongols, Tartars, and other Asian rulers to Russia and Europe also contributed to the spread of yogurt and fermented milk to other parts of the world. Yogurt is now a very popular product and an important part of the diet in Europe, Australia, and many other parts of the world. Yogurt and fermented milk are now being manufactured commercially in most major countries. Traditional ways of yogurt production for daily consumption are still being followed in many regions. Table 1.1 shows the different names and kinds of yogurts, and yogurt-like fermented beverages found around the world.

<table>
<thead>
<tr>
<th>Traditional Name</th>
<th>Country</th>
<th>Traditional Name</th>
<th>Country</th>
</tr>
</thead>
<tbody>
<tr>
<td>Busa</td>
<td>Turkestan</td>
<td>Mezzoradu</td>
<td>Sicily</td>
</tr>
<tr>
<td>Cieddu</td>
<td>Italy</td>
<td>Roba</td>
<td>Iraq</td>
</tr>
<tr>
<td>Dahi/Dadhi/Dahee</td>
<td>India, Bangladesh, Nepal</td>
<td>Skyr</td>
<td>Iceland</td>
</tr>
<tr>
<td>Filmjolk/Fillunke/Filbunk/Surmelk/</td>
<td>Scandinavia</td>
<td>Taettem-jolk/Tettemelk</td>
<td>Hungary</td>
</tr>
<tr>
<td>Gioddu</td>
<td>Sardinia</td>
<td>Tarho</td>
<td>Greece</td>
</tr>
<tr>
<td>Jugurt/Eyran</td>
<td>Turkey</td>
<td>Tiaourti</td>
<td>Balkan Mountains</td>
</tr>
<tr>
<td>Katyk</td>
<td>Transcaucasia</td>
<td>Urgotnic</td>
<td>Finland</td>
</tr>
<tr>
<td>Kissel Mleka</td>
<td>Balkans</td>
<td>Villi</td>
<td>Rest of the world</td>
</tr>
<tr>
<td>Leben/Leban</td>
<td>Lebanon and some Arab countries</td>
<td>Yogurt/Yogurt/Yaort</td>
<td>(“Y” is replaced by “J” in some instances)</td>
</tr>
<tr>
<td>Mast/Dough</td>
<td>Iran and Afghanistan</td>
<td>Yogurt/Yaourt/Yahourth/Yogurt/Yaghourt</td>
<td>Egypt and Sudan</td>
</tr>
<tr>
<td>Mazun/Matzoen</td>
<td>Armenia</td>
<td>Zabady</td>
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</tbody>
</table>

The subtropical warm temperature played a major role in the fermentation of milk in the Middle Eastern belt. The subtropical climate of about 40°C is optimal for bacterial culture growth in milk resulting in coagulation of milk and formation of yogurt, and other fermented milk beverages and products. This uncontrolled natural fermentation, without the addition of a known starter culture, would yield products with different characteristics in different conditions and locations. This led to the formation of insipid product, with irregular coagulum filled with air (Kosikowski and Mistry, 1997). Yogurt and other fermented products vary in their characteristics, taste, texture, composition, and health properties based on the type of milk used and its composition, and the nature and activity of fermenting bacteria (Chandan and Kilara, 2013).

Traditional production of yogurt by nomads was carried out in animal skin pouches. The skin allowed enhanced removal of whey, thus making this yogurt very thick and high in solid content and lactic acid. Such concentrated yogurt is thus characterized as condensed yogurt. It possesses relatively low amounts of whey. This kind of condensed yogurt is popular in Armenia, where concentrated Armenian yogurt, mazun, becomes a product called than. In South Asian countries such as India and Nepal, yogurt is concentrated by keeping it in an earthenware vessel. This earthenware keeps the yogurt cool, and the evaporation through the pores of the vessel increases the lactic acid concentration and solids content in the product. Salt is added to the product to enhance the shelf life of fermented yogurt.

Another condensed yogurt is called Greek-style yogurt (Kilara and Chandan, 2013). Traditionally, it was made in Greece by straining whey from yogurt in a pouch of cloth. The popularity of Greek yogurt is attributed to the healthy appeal of the high protein content (2–2.5 times that of regular yogurt). In India, dahi (a variant of yogurt) has been immensely popular fermented dairy food for centuries. Shrikhand is obtained by straining dahi through cloth. This product is consumed as a snack or dessert in Western India. There is a striking resemblance between Greek yogurt and shrikhand. They differ only in the type of culture used for fermentation.

Salting of concentrated yogurt varies in different types of yogurts in Turkey. To enhance the shelf life of the product and to maintain the quality, the salted products were often sun-dried. These dried yogurt balls were then stored in glass jars and covered in olive oil. In other countries like Iran, Iraq, Lebanon, and Turkey, wheat flour was rubbed on the surface of the dried yogurt to maintain the quality for almost an indefinite period; this product is commonly known as kishk. With the widespread usage of refrigerators, the shelf life of different kinds of yogurt increased and the application of these traditional methods of preserving yogurt slowly declined.

Nobel laureate Elie Metchnikoff at the Pasteur Institute in Paris first proposed a scientific rationale for the beneficial health effects of the yogurt bacteria at the beginning of the last century. In his article entitled “The Prolongation of Life” from the use of yogurt bacteria, he hypothesized that *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* control the infections caused by enteric pathogens and regulate toxemia, both of which play a major role in ageing and mortality. He also related the longevity and good health of the Bulgarian peasants to their high consumption of yogurt and other fermented milk products. It was later discovered that the common yogurt starter bacteria *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* were unable to implant in the intestine. In the 1900s, Moro isolated *L. acidophilus* from human infant feces. More emphasis was then given to *L. acidophilus* for its therapeutic properties, making it a preferable adjunct organism. This observation provided a great impetus to the manufacture and consumption of yogurt (Prajapati and Nair, 2003).
1.3 GLOBAL POPULARITY OF YOGURT

Yogurt has been a remarkably successful dairy product of recent times. Over the past few decades the popularity of yogurt has been increasing worldwide. This is largely attributed to the research findings that have highlighted the health benefits associated with yogurt consumption in various ethnic groups around the world. Apart from the increasing awareness about the health benefits of yogurt, product innovation, and availability of different flavors and kinds of yogurt have contributed significantly to the enhanced consumption of yogurt over the past few decades.

The popularity of yogurt is primarily due to its many health benefits (Shah and Champagne, 2016; Chandan, 2015). A considerable amount of evidence has accumulated for some benefits such as improved lactose tolerance. Apart from the nutritional benefits, fermented milk products also have some physiological benefits such as antimicrobial activity and activity against gastrointestinal infections, anticancer effects, and reduction in serum cholesterol and immune system stimulation (Holm, 2003; Chandan and Nauth, 2012; Shah, 2013a,b, 2014, 2015; Shah et al., 2013; El-Abbadi et al., 2014; Chandan, 2015; Madjd et al., 2016).

Milk protein is known for its high nutritional value, mainly attributed to its biological value, net protein utilization, and high protein efficiency ratio (Chandan, 2016). Milk proteins are of excellent quality due to the high amount of essential amino acids present in caseins and whey proteins (α-lactalbumin and β-lactoglobulin). Yogurt and other fermented products contain higher protein, mainly due to supplementation with skim milk solids (around 2%-3%). This implies that yogurt may be a better source of protein as compared with yogurt beverages or other fermented milk drinks. Heat treatment of milk and the action of starter bacteria during yogurt production cause the breakdown of milk protein, leading to increased level of soluble proteins, free amino acids, and nonprotein nitrogen. Greek-style yogurt provides even more protein per serving as compared to regular yogurt.

Since popularity of yogurt has been based on its health attributes, unprecedented numbers of research publications on this topic have appeared in the literature. Some recent findings are discussed next. The image of health attributes is changing rapidly from anecdotal evidence to scientific clinical evidence.

1.4 HEALTH BENEFITS OF YOGURT

Fermented milk products have been considered from ancient times to enhance intestinal health. Even before scientific studies documented this benefit, fermented milk products were used to alleviate diarrhea caused by infection of pathogenic bacteria. The fermented food products enhance the metabolic activity and the composition of gut microflora. This helps maintain a healthy microbial balance in the gut, which is usually altered during gastrointestinal disorders, stress, use of antibiotics, and from other diseases. The yogurt starter bacteria produce bacteriocins, which are antimicrobial agents produced to inhibit the contaminant pathogenic bacteria. Bacteriocins are proteins or peptides (Nandkumar and Talapatra, 2014). Several lactobacilli also produce hydrogen peroxide, which is believed to be an antimicrobial agent. L. delbrueckii ssp. bulgaricus produces bacteriocins, including “bulgarican,” which has shown broad-spectrum antibacterial activity. Antimicrobial compounds isolated from skim milk cultures of L. delbrueckii ssp. bulgaricus and S. thermophilus have shown activity against a range of organisms including Salmonella, Shigella, Escherichia coli, and Pseudomonas (Dave and Shah, 1997).
Milk is an excellent medium to carry or generate live and active cultured dairy products (Chandan, 1999; Chandan and Nauth, 2012; Chandan and Shah, 2013; Shah, 2007, 2013a,b). The buffering action of the milk proteins keeps the cultures active during their transit through the gastrointestinal tract. In general, worldwide consumption of fermented milk products has increased due to their high nutritional profile, unique flavor, desirable texture, and remarkable safety against food-borne illness.

Since yogurt is made from milk, the benefits of yogurt consumption include the nutritional value associated with dairy nutrients. Furthermore, the health properties of milk components are enhanced by culturing of milk, ascribed to live cultures and products of metabolic activities of yogurt cultures (Chandan, 1999). The live and active cultures in significant populations are known to produce physiological effects for the consumer. Health benefits of milk have been discussed in another publication (Chandan, 2016). Of particular consideration is the health of bones and teeth. Milk provides bioavailable calcium, phosphorus, magnesium, and protein, which are essential for healthy bone growth and development. Consumption of adequate amounts of milk and its products from early childhood and throughout life results in strong bones and protects them from brittle bone disorder (osteoporosis) in later life. Both calcium and phosphorus assist in the development and maintenance of healthy teeth. Casein, the main protein of milk, forms a thin layer on teeth enamel, which has a protective effect on teeth against food acid attack. It also reduces the cariogenic foods on the teeth. Thus, health benefits of yogurt are cumulative due to milk nutrients and fermentation-derived metabolites as well as large bacterial mass accumulated as the result of fermentation.

Probiotics may be defined as a food or supplement containing concentrates of defined strains of living microorganisms that on ingestion in certain doses exert health benefits beyond inherent basic nutrition. Probiotics and associated ingredients add an attractive dimension to cultured dairy foods for effecting special functional attributes (Chandan, 2007). Cultures associated with health benefits are yogurt bacteria *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus*, other lactobacilli and bifidobacteria (Chandan and Nauth, 2012; Chandan and Shah, 2013). Yogurt organisms possess several documented health attributes. To bolster probiotic function, most commercial yogurt is generally supplemented with various species of genera *Lactobacillus* and *Bifidobacterium* (Shah, 2011a,b). Yogurt starter bacteria, *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus*, are also now considered to act as probiotics because of their health-promoting effects. In particular, yogurt bacteria have been scientifically demonstrated to assist in lactose digestion, reduce or prevent diarrhea episodes, and strengthen immune defenses of the host. They are now reported to persist and remain viable throughout the human gastrointestinal tract. The continuous ingestion of live products ensures abundant numbers to maintain their functional status. Even with intestinal isolates such as *L. acidophilus*, it is necessary to dose regularly rather than to assume that a few doses will allow the organisms to colonize the gut permanently.

Several hundred bacterial species inhabit the distal regions of the human digestive tract. Their population exceeds the total cell counts in the human body. Functions of the 100 trillion bacteria inhabiting the colon include modulation of cell growth and differentiation, antagonistic activity against pathogens and other infections, immune stimulation of gut-associated lymphoid tissue, reduction of blood lipids, and biosynthesis of vitamins (Granato et al., 2010; Franz et al., 2013; Donovan and Shamir, 2014). In healthy individuals, the colonies of diverse gut bacteria exist in equilibrium. Factors such as stress, age, gastrointestinal disturbances, and antibiotic therapy are known to upset the balance of gut microflora, and the resultant malfunctioning of their digestive and metabolic effects. Probiotics help in restoring the balance. Diet rapidly and reproducibly alters the human gut microbiome (Chandan, 2014, 2015, 2016; Chandan and Nauth, 2012). Long-term dietary intake influences the structure and activity of the
microorganisms residing in the human gut. David et al. (2014) showed that the short-term consumption of diets composed entirely of animal or plant products alters microbial community structure and overwhelms interindividual differences in microbial gene expression. The animal-based diet increased the abundance of bile-tolerant microorganisms (Alistipes, Bilophila, and Bacteroides) and decreased the levels of Firmicutes that metabolize dietary plant polysaccharides (Roseburia, Eubacterium rectale, and Ruminococcus bromii). Microbial activity mirrored differences between herbivorous and carnivorous mammals, reflecting trade-offs between carbohydrate and protein fermentation. Food-borne microbes from both diets transiently colonized the gut, including bacteria, fungi, and even viruses. Finally, increases in the abundance and activity of Bilophila wadsworthia on the animal-based diet support a link between dietary fat, bile acids, and the outgrowth of microorganisms capable of triggering inflammatory bowel disease. In concert, these results demonstrate that the gut microbiome can rapidly respond to altered diet, potentially facilitating the diversity of human dietary lifestyles.

Major benefits of consuming fermented milk are documented in the literature and briefly discussed in the next section.

1.4.1 TYPE 2 DIABETES
Regular consumption of dairy products can reduce the risk of type 2 diabetes. It is believed that calcium and magnesium content are responsible for the effect.

1.4.2 OBESITY
Gut health and obesity appear to be related. Re et al. (2006) reported that microbial populations in the guts of lean and obese people are different. After obese people lose weight, their gut microflora reverts to that of lean persons. Studies have shown that consumption of milk products as a part of calorie-controlled diet is associated with increased weight loss, especially from the abdomen.

1.4.3 GASTROINTESTINAL BENEFITS
1.4.3.1 Lactose Digestion
The enzyme lactase is responsible for lactose digestion. Although it is present in the suckling infant but it may disappear after weaning. Normally, the disaccharide lactose is hydrolyzed to glucose and galactose by lactase and subsequently absorbed in the small intestine. In areas of the world where milk is not a staple food, lack of the enzyme lactase causes no problems. Lactase intolerance refers to incomplete digestion of lactose resulting in a flat or low rise in blood sugar following ingestion of lactose in a clinical lactose intolerance test. Lactase is a constitutive, membrane-bound enzyme located in the brush borders of the epithelial cells of the small intestine. In lactose-intolerant individuals, lactase activity is impaired resulting in intact lactose entering the colon where it is fermented by inherent microflora to generate organic acids, carbon dioxide, methane, and hydrogen. The fermentation products together with the osmotically driven excessive water drawn into the colon are primarily responsible for abdominal pain, bloating, cramps, diarrhea, and flatulence. It has been known for some time that lactose-deficient subjects tolerate lactose in yogurt better than the same amount of lactose in milk. Some of the lactose content of milk is metabolized during fermentation. The majority of lactose remains intact and it is broken down to easily absorbable glucose and galactose by the constituent lactase enzyme of the
There is strong clinical evidence in the literature that by consuming yogurt there is marked improvement in lactose utilization (Savaiano, 2014; Prentice, 2014). Human studies have clinically demonstrated that following feeding yogurt to lactose-intolerant individuals increased lactase activity in the small intestine of humans is observed.

### 1.4.3.2 Overall Digestion and Nutritional Enhancement

Yogurt and other fermented milk products are also rich in vitamins A, B₂, B₁, B₆, and B₁₂ (Chandan, 2016). Lactic acid bacteria (LAB) have also been shown to possess the ability to synthesize B vitamins, such as folic acid (Le Blanc et al., 2013) during fermentation. On the other hand, some of the B vitamins (such as B₁₂) decrease in fermented products, as the LAB may require these during fermentation. The amount of vitamin A, which is a fat-soluble vitamin, depends on the level of fat in the product. In general, the content of vitamins in yogurt is higher than in milk.

Yogurt is easier to digest because it assists in lactose and protein digestion. The heating step during yogurt processing denatures whey proteins making them less allergenic. Subsequent fermentation process results in partial digestion of casein and whey protein by the protein hydrolyzing enzymes of yogurt culture. Compared to milk, lactic acid content and vitamin B content of yogurt further assist in overall digestion.

### 1.4.3.3 Prevention and Treatment of Diarrhea

Establishment of probiotics in the gastrointestinal tract may provide prophylactic and therapeutic benefits against intestinal infections. Probiotics may have a role in circumventing traveler’s diarrhea. Yogurt supplemented with probiotic organisms reduces the duration of certain types of diarrhea. Yogurt with probiotics has been recommended to replace milk during treatment of diarrhea treatment because it is tolerated better than milk. A double-blind study has shown that only 7% of infants receiving probiotic formula containing *Bifidobacterium bifidum* and *S. thermophilus* develop diarrhea against 31% incidence in placebo group (Saavedra et al., 1994). Guerin-Danan et al. (1998, 2001) demonstrated protection from rotavirus-associated diarrhea by feeding milk fermented with *L. casei* DN-114 001 in suckling rats. They extrapolated similar protection to young children. Gutierrez-Castrellon et al. (2014) reported that in healthy schoolchildren attending day care centers, daily consumption of *Lactobacillus reuteri* DSM 17938 had a significant effect in reducing episodes and duration of diarrhea and respiratory tract infection, with consequent cost savings for the community.

### 1.4.3.4 Irritable Bowel Syndrome

In a 28-strong cohort study, subjects suffering from irritable bowel syndrome (IBS) with constipation were asked to consume milk fermented with *Bifidobacterium lactis*, *S. thermophilus*, *L. bulgaricus*, and *Lactococcus lactis* (FM) for 4 weeks. Acidified milk acted as a placebo. The subjects consuming FM displayed positive changes in gut microbiome induced by the probiotics. The IBS sufferers normally show lower levels of butyrate-producing microorganisms in their gut. However, the subjects on FM restored the butyrate-producing bacteria. Butyrate is recognized as beneficial in gut health (Viega et al., 2014).

### 1.4.3.5 Fatty Liver Disease

The gastrointestinal tract microbiota appears to influence a major hepatic complication, nonalcoholic fatty liver disease (NAFLD) (Mohammadmoradi et al., 2014). Recent studies have proposed that
probiotics may have beneficial effects on the treatment/prevention of NAFLD and other hepatic complications due to their ability to augment intestinal barrier function, prevent production of lipopolysaccharides (LPSs), and modulate the immune system. Nabavi et al. (2014) reported that consumption of probiotic yogurt containing *L. acidophilus* La5 and *B. lactis* Bb12 resulted in reductions of 4.67% and 5.42% in serum levels of indicator enzymes of fatty liver alanine aminotransferase and aspartate aminotransferase, respectively, compared with the control group. In addition, they observed a reduction of 4.1% and 6.92% in total cholesterol and serum LDL cholesterol, respectively, in the probiotic group. They concluded that probiotic yogurt consumption improved hepatic enzymes, serum cholesterol, and LDL cholesterol levels in the subjects studied. Accordingly, probiotic yogurt may be useful in management of NAFLD factors. Ma et al. (2013) reported that probiotic therapy can reduce liver aminotransferase, total cholesterol, TNF–α, and improve insulin resistance in NAFLD patients.

### 1.4.4 REDUCTION IN CARDIOMETABOLIC DISEASES AND SERUM CHOLESTEROL

Research data obtained from clinical studies suggests that yogurt consumption as a part of a healthy diet may be beneficial in the prevention of cardiovascular diseases (Astrup, 2014; Marette and Pickard-Deland, 2014). Probiotics help to partially reduce cholesterol levels circulating in blood. Some studies have indicated a modest lowering of serum cholesterol in subjects consuming milk fermented with *L. acidophilus*, *Lactobacillus rhamnosus* GG and yogurt cultures. Because of bioavailability of inherent calcium and potassium, yogurt prevents high blood pressure. Yogurt appears to reduce risk of metabolic syndrome and related diseases like diabetes. Beserra et al. (2014) published a meta-analysis study indicating potential for reduction of obesity-related issues by prebiotics and synbiotics. They were associated with reductions in total and LDL cholesterol and with improvements in triglyceride and HDL cholesterol levels in diabetics. Rodríguez-Figueroa et al. (2013) determined antihypertensive and hypolipidemic effects of milk fermented by specific *L. lactis* strains in spontaneously hypertensive rats (SHR). The SHR were fed ad libitum milk fermented by *L. lactis* NRRL B-50571, *L. lactis* NRRL B-50572, Captopril (40 mg/kg of body weight, Sigma-Aldrich Co., St. Louis, MO) or purified water for 4 weeks. The investigators reported that *L. lactis* fermented milk presented a significant blood pressure-lowering effect. No significant difference was noted among milk fermented by *L. lactis* NRRL B-50571 and captopril by the second and third week of treatment. Additionally, milk fermented by *L. lactis* strains modified SHR lipid profiles. Milk fermented by *L. lactis* NRRL B-50571 and B-50572 was able to reduce plasma low-density lipoprotein cholesterol and triglyceride contents. Thus, milk fermented by *L. lactis* strains may be a coadjuvant in the reduction of hypertension and hyperlipidemia and may be used as a functional food for better cardiovascular health. This study is interesting in that milk fermented with *L. lactis* demonstrated probiotic properties similar to *Lactobacillus* and *Bifidobacterium*.

Beltran-Barrientos et al. (2016) reviewed antihypertensive effects of fermented milk. They reported that milk fermented with *L. helveticus* displays blood pressure and heart rate lowering properties. This benefit is ascribed to the bioactive peptides generated by the culture.

### 1.4.5 BONE STRUCTURE AND INTEGRITY

Milk is one of the richest sources of calcium, and absorption of calcium from milk is better than from other products. Although fermentation does not alter the mineral content, the utilization of some
minerals (such as calcium, phosphorous, and iron) in human body is better utilized from fermented milk as compared to that from milk alone. It is believed that peptides such as phosphopeptides, released by the breakdown of casein, may accelerate mineral absorption. Lactic acid was suggested to play a role in calcium content in bone and in strength of bone. These and more studies have shown that absorption of calcium is higher from fermented milk as compared with whole unfermented milk. The health benefits of calcium from milk also include prevention of osteoporosis and regulation of bone metabolism. However, sodium and potassium, which are also present in considerable amounts in yogurts, make it inappropriate for consumption by infants.

Yogurt consumption in rats showed increased feed efficiency and increased body weight as compared to the milk. It has also been reported that the growth-promoting effect is attributed to *S. thermophilus*, which also improved mineral bioavailability from the product. Yogurt has also been found to have higher digestibility than milk, which may be due to decreased protein particle size, increased soluble nitrogen, and free amino acids released during heat processing of milk and fermentation. One of the primary functions of calcium along with protein is to provide strength and structural properties to bone and teeth. The major source of dietary calcium is dairy products, which are excellent sources of bioavailable calcium (Zittermann, 2011). Addition of lactic acid to unfermented yogurt as well as regular fermented yogurt display improved bone mineralization as compared to the unfermented yogurt. It is postulated that the acidic pH due to added lactic acid or naturally contained in fermented yogurt converts colloidal calcium to its ionic form and allows its transport to the mucosal cells of the intestine. Yogurt consumption improves bone health and reduces the risk of fractures in later life (Prentice, 2014; Morelli, 2014).

### 1.4.6 IMMUNO-MODULATORY EFFECTS

The starter bacteria used for yogurt production can survive in the gastrointestinal tract and have been detected in feces. This indicates that the starter bacteria may contribute health-promoting roles of yogurt and other fermented milk beverages. The intestine is the largest immune system in the human body and the intestinal system protects the body against bacterial and viral infection and cancer and allergies. The gastrointestinal tract includes about 80% of our antibody-producing cells and forms a vital part of our defense system. The intestinal tract, which harbors most of the intestinal microflora, protects against infections and also plays a role in systemic immunological function. It is believed that the LAB in the intestine play a crucial role in the stimulation of immune system by modifying function of immune cells and by activation of macrophages and “natural killer” cells. They also stimulate certain cytokines, such as IL-6 and IL-10. The yogurt starter bacteria are also reported to produce γ-interferon by T cells. Yogurt consumption has been associated with stimulation of cytokine production in blood cells and activation of macrophages.

The ability of the gastrointestinal system to function properly is dependent on the interaction of the intestinal microbes, the intestinal barrier, and the immune system. Round and Mazmanian (2009) reviewed the role of gut microbiota in shaping intestinal immune response during disease and health. They found that disturbance in the bacterial microbiota results in dysregulation of adaptive immune cells, leading to irritable immune disease. An interesting development in recent years has been the finding that lactobacilli administered by mouth can stimulate macrophage activity against several different species of bacteria (Brassart and Schiffrin, 1997; Lee et al., 1996; El-Abbadi et al., 2014). For example, *L. casei* given to mice increased phagocytic activity. Lactobacilli injected intravenously are reported to survive in the liver, spleen, and lungs and enhance the natural killer cell activity.
Makino et al. (2006, 2016) reported that consumption of yogurt cultured with *L. delbrueckii* ssp. *bulgaricus* OLL1073R-1 possessed enhanced natural killer cell activation. This activity was related to exopolysaccharides produced by the culture.

### 1.4.6.1 Antiallergic Effect

Singh et al. (2013) reported that a daily dose of a probiotic strain *B. lactis* NCC2818 may help manage allergic responses during pollen season. Eight weeks of the probiotic intake significantly reduced the symptoms of hay fever and allergic rhinitis. Stefka et al. (2014) reported that alterations in trillions of gastrointestinal tract inhabitants (microbiota) influence allergic response to food. They have identified a specific bacterial community that protects against sensitization by regulating epithelial permeability food allergens. Song et al. (2016) published a study showing that yogurt made with *Lactobacillus plantarum* L67 has potential to prevent allergy-related immune disorders.

### 1.4.7 CONTROL OF INFECTIONS

Barker et al. (2015) reported a study related to infection in elderly patients. *Clostridium difficile* is a pathogen of rapidly increasing public health importance. Probiotics may have potential as adjunctive therapeutic agents for *C. difficile* infection.

#### 1.4.7.1 Prevention and Cure of Vaginitis

The vaginal microflora changes drastically during bacterial infection. Bacteria of genera *Escherichia*, *Proteus*, *Klebsiella*, and *Pseudomonas* along with yeast, *Candida albicans*, are recognized as etiological agents in urinary tract infection among adult women. It has been shown that the normal urethral, vaginal, and cervical flora of healthy females can competitively block the attachment of uropathogenic bacteria to the surfaces of uroepithelial cells. *Lactobacillus* strains supplemented in the diet or directly applied are reported to coat the uroepithelial wall and prevent the adherence of uropathogens. Milk fermented with yogurt cultures and *L. casei* influenced the intestinal microflora of infants (Guerin-Danan et al., 1998). In another study, Martinez et al. (2009) conducted a randomized, double-blind, placebo-controlled study with 64 Brazilian women diagnosed with bacterial vaginosis (BV). The subjects were randomly assigned to receive a single dose of tinidazole (2 g) supplemented with either two placebo capsules or two capsules containing *L. rhamnosus* GR-1 and *L. reuteri* RC-14 daily. After 28 days of treatment, the probiotic group had a significantly higher cure rate of BV (87.5%) than the placebo group (50%). In addition, the vaginal microbiota of the probiotic women group was assessed to be normal (75% vs. 34%) than in the placebo group. In another publication, Beerepoot et al. (2012) studied postmenopausal women with recurrent urinary tract infections. *L. rhamnosus* GR-1 and *L. reuteri* RC-14 did not meet the noninferiority criteria in the prevention of urinary tract infection when compared with trimethoprim-sulfamethoxazole, but comparatively the lactobacilli did not cause increase in antibiotic resistance.

#### 1.4.7.2 Reduction of Duration of Common Cold

Makino et al. (2006) reported that milk fermented with *L. delbrueckii* ssp. *bulgaricus* OLL1073R-1 reduced the risk of catching common colds and flu in human trials. A recent review supports that consuming yogurt with probiotics decreases the duration of common cold and upper respiratory tract infections (King et al., 2014).
1.4.8 ANTICARCINOGENESIS

Cancer is one of the leading causes of deaths worldwide. Several studies have suggested that environmental and lifestyle factors, such as diet, play a significant role in altering the risk of cancer incidence. For instance, consumption of cooked/barbequed red meat and low consumption of fiber are reported to play a major role. Several genotoxic compounds or metabolites may be formed, which in turn cause colorectal cancer. Bacterial enzymes such as $\beta$-glucuronidase generate these carcinogenic products, except LAB and probiotics.

LAB and several fermented products have been reported to possess anticarcinogenic properties. These LAB suppress the bacterial enzymes, which may produce carcinogenic metabolites, and also reduce the intestinal pH. The administration of LAB-fermented products, including yogurt, has shown to exert beneficial effects against cancer (de LeBlanc, 2014). Antiproliferative effect of fermented milk on the growth of human breast cancer line has also been demonstrated. These anticancer activities of yogurt are attributed to stimulation of immune functions of the body as well as improvement in intestinal microflora population. The proposed mechanism of the anticarcinogenic effect of LAB is by the removal or inhibition of the sources of procarcinogens or the enzymes leading to their formation. During growth of LAB in milk short-chain fatty acids are produced, which also inhibit the release of carcinogenic compounds by inhibiting the enzyme activities. The second proposed mechanism involves improved balance of intestinal microorganisms, normalized intestinal permeability by delaying toxin absorption, and strengthening the intestinal barrier mechanisms.

*Bifidobacterium* and *Lactobacillus*, especially *L. acidophilus*, have been shown to have powerful anticarcinogenic features, which are active against certain tumors. An epidemiological study reported a positive correlation between consumption of probiotics and prevention of colon cancer. Several reports suggest prevention of cancer initiation by various probiotics by reducing the fecal procarcinogenic enzymes nitroreductase and azoreductase (Lee et al., 1996). A recent study (Elfahri et al., 2016) showed that protein-derived bioactive peptides of skim milk fermented with *L. helveticus* exerted antioxidant and anticarcinogenic properties.

1.4.9 PRODUCTION OF VITAMINS AND CONTROL OF PATHOGENIC ORGANISMS

Probiotics also produce some of the B vitamins including niacin, pyridoxine, folic acid, and biotin. In addition, they produce antibacterial substances that have antimicrobial properties against disease-causing bacteria. Acidophilin produced by *L. acidophilus* is reported to inactivate 50% of 27 different disease-causing bacteria. Children with salmonella poisoning and shigella infections were cleared of all symptoms using *L. acidophilus*. *B. bifidum* effectively kills or controls *E. coli*, *Staphylococcus aureus*, and *Shigella*. Acidophilus is also reported to control viruses such as herpes (Chandan and Shah, 2013). Pan et al. (2016) reported on the changes in gastric microbiota induced by *Helicobacter pylori* infection and preventative effects of *L. plantarum* ZDY 2013.

1.4.10 WEIGHT MANAGEMENT

Several studies have been conducted to determine the role of milk and fermented milk including yogurt on human body composition and weight control. This work has suggested that yogurt consumption is associated with a reduced risk of weight gain and obesity (Astrup, 2014; Jacques and Wang, 2014). Some studies have suggested yogurt may help in reducing or controlling weight and waist
circumference in men and women (Kelishadi et al., 2014). Work of Kadooka et al. (2010) has shown that the probiotic *Lactobacillus gasseri* (strain LG2055/SBT2055) lowered abdominal adiposity, body weight, and other measures, suggesting its beneficial influence on metabolic disorders.

### 1.4.11 SKIN HEALTH

Kimoto-Nira et al. (2014) reported that in a randomized double-blind study, consumption of milk fermented with *L. lactis* H61 resulted in beneficial effects on the skin of young women. The literature cites numerous benefits of consuming probiotics. To resolve confusion and concern around probiotic data and use of the term *probiotic*, the expert panel of the International Scientific Association of Probiotics and Prebiotics has agreed on core benefits of certain probiotics (Hill et al., 2014). The panel concluded that the general benefit of supporting a healthy digestive tract was reinforced by evidence gathered on a large number of different probiotic strains representing commonly studied species. Certain strains of the *Bifidobacterium* and *Lactobacillus* species were recognized for gut health. The core effects on gut physiology and health were based on a body of available research, including high-quality meta-analyses, on a diversity of clinical end points (such as infectious diarrhea, antibiotic-associated diarrhea, gut transit, IBS, abdominal pain and bloating, ulcerative colitis, and necrotizing enterocolitis). The panel stated that the immune benefits are probably more strain specific. Other less-researched benefits including the health of the reproductive tract, oral cavity, lungs, skin and gut–brain axis were promising, but could not yet be shared across the whole class of probiotics. The panel confirmed that the current World Health Organization definition of a probiotic as “live microorganisms that, when administered in adequate amounts, confer a health benefit on the host” was relevant and acceptable.

### 1.5 POPULAR VARIETIES OF YOGURT

The first commercial production of yogurt in Europe was undertaken by Danone in 1922 in Madrid, Spain. In the following few decades, especially since the 1950s, there was immense research and rapid advancements in the technology of yogurt and understanding its properties. Today there are numerous varieties and flavors of yogurt available in the market.

Various varieties of yogurt are discussed in detail (Chandan and Kilara, 2013).

#### 1.5.1 PLAIN YOGURT

Plain yogurt contains no sugar or sweeteners. It is made from dairy ingredients and may be cup fermented or vat fermented. It may be consumed as such allowing the consumer to add fruits of their choice. In some cases, savory yogurt may be prepared by adding vegetables or other food ingredients. In some applications, plain yogurt may be substituted for sour cream.

#### 1.5.2 FRUIT OR FLAVORED YOGURT

Fruit yogurt is one of the most favorable types of yogurt in the current market. Flavored yogurts are prepared by adding sugar and fruit flavorings to plain yogurt. The production of fruit yogurt is very similar to that of the normal yogurt. The first three steps, i.e., standardization,
homogenization, and heat treatment, are similar. Addition of fruit may be after heating and before fermentation in set-style fruit yogurt, or after fermentation in stirred-style fruit yogurt. Stirred-style fruit yogurt, also known as the “Swiss-style” yogurt, is more popular in Australia and the United States. The enhanced color, taste, and texture of fruit yogurt are generally more appealing to a vast majority of customers.

Fruit flavorings and preparations are prepared according to the variety of yogurt (Chandan and Kilara, 2013; O’Rell and Chandan, 2013a,b). The protocols approved for fruit yogurt state the minimum fruit level as 5%, with an exception for passion fruit (3.5%). Sundae-style yogurt is prepared by layering 15%–18% of fruit puree or syrup on the bottom of the containers and pouring warm inoculated mix over the puree or syrup, followed by sealing the containers and incubating. The temperature of the inoculated milk usually is 46°C. However, the temperature drops to 42°C when the fruit is covered. The fermentation is carried out at this temperature for 4 h followed by refrigeration. The coagulum remains firmly set. The fruit in the product is mixed with the yogurt gel by consumers before consumption.

Swiss- or stirred-style yogurts are prepared by blending fruit puree, fruit preserve, honey, sucrose, or glucose into bulk-prepared fresh plain yogurt. The blending is usually carried out at 15°C. Vanilla essence may be incorporated in the yogurt mix prior to incubation. Sugar could be added before or after incubation depending on the amount to be added. High sugar concentration (8% or more) may interfere with the growth of starter bacteria. Ravula and Shah (2000) have demonstrated inhibition of starter bacteria at high levels of added sweetener in yogurt mix preparation, thereby highlighting the importance of monitoring the content of additives.

Fruit purees or fruit preserves are added at the rate of 15%, however, higher amounts are reported to be used by some manufacturers. Since the coagulum is broken during blending, plain yogurt is usually prepared with higher level of stabilizer (0.7%) than normal (0.3%) to achieve high viscosity. The product after mixing with fruit is chilled to 4°C. Since flavored Swiss-style yogurt contains up to 0.7% stabilizer, the product can be frozen, unlike plain yogurt.

Stabilizers that are commonly used include gum, carrageenan, gelatin, carboxymethyl cellulose, and modified starch or pectin or blends of these. Stabilizers are hydrophilic polysaccharides that help prevent whey syneresis as they absorb water. The other permitted additives to fruit yogurt include sugars, vegetable gum, gelatin, honey, polydextrose, and other modified starches in defined amounts. Stabilizers also help improve viscosity and give proper mouthfeel. A typical vanilla-flavored yogurt contains 3.4% fat, 4.9% protein, 9.7% total sugar, and 0.7% stabilizer. The lactose content may vary from 3% to 5%, however, extra sugar (up to 15%) is usually added. This increases caloric content of flavored yogurt significantly.

The fruit should be just ripe when initially processed, as unripe fruit being tough, may not impart the required color and flavor. Also, overripe fruit may be too soft to withstand the processing steps, or may have increased tendency to harbor intrinsic yeasts and contaminants. The fruit is usually finely chopped, providing a more appealing look to the consumers. The fruit should also meet the required standards relating to its texture and quality. The viscoelastic characteristic of the fruit should be adequate to ensure that it remains suspended in the yogurt and does not form a gel-like suspension during storage. Sugar content in fruit preparation should be within the range of 0% to 60% of sucrose. Furthermore, the desired sugar/acid ratio, and the amount of sweetener, should also be carefully monitored.

Addition of fruits to fermented milk reduces the viscosity due to reversible breakdown of structure, which rebuilds to a greater extent or lesser extent with time. However, if the shear is too high, then an
irreversible breakdown of the structure may occur. In such cases, a stabilizer is generally introduced, which reinforces the thixotropic nature of the coagulum. Furthermore, the mixing of the fruit and yogurt phases should be done carefully to pose minimal structural damage to coagulum.

1.5.3 GREEK YOGURT OR STRAINED YOGURT

Its significantly higher protein content propels the popularity of Greek yogurt. Greek yogurt is conventionally prepared by straining the fermented cooled yogurt curd to remove whey using a cloth bag until a desired solids level is attained. Sometimes a crust on the surface of the container characterizes traditional Greek yogurt, which usually consists of fat and proteins of the serum. The formation of this crust is due to use of nonhomogenized milk. The presence of typical crust on the surface characterizes the original/traditional Greek yogurt. For commercial purposes, natural straining has been replaced by the use of centrifugation and filtration. The production of large quantities of acid whey presents both economic and environmental challenges, and researchers are coming up with innovations to solve the problem (Desai et al., 2013).

Greek yogurt, strained yogurt, or labneh possesses a thicker consistency than unstrained yogurt. More details on Greek yogurt are available in a publication by Kilara and Chandan (2013). Greek yogurt is often made from milk that has been enriched for protein by membrane filtration (ultrafiltration) of milk, or by adding powdered milk protein concentrate to milk. In Europe and North America, it is often made by mechanical separation of whey from fermented low-fat or fat-free yogurt. In Scandinavia, particularly Iceland, a similar product, skyr, is produced.

Greek yogurt is also widely consumed in Eastern Mediterranean, Near Eastern, Central Asian, and South Asian countries. Straining process to remove whey varies from manufacturer to manufacturer and even nonfat varieties of Greek yogurt are much thicker, richer, and creamier than yogurts that have not been strained.

In the United States, Canada, and Western Europe, strained yogurt has become increasingly popular compared to unstrained yogurt. Greek yogurt is reported to now have 50% share of the yogurt market in the United States. Part of the success of Greek yogurt may be due to its healthy alternative perception (double the protein content, all-natural ingredients, and low sugar content). Greek yogurt forms the basis for production of beverages, frozen yogurt, cheesecake, and other foods. It may be blended with fruit preparations like regular yogurt. Some of the Greek yogurt brands in North America include Chobani, Dannon Oikos, FAGE, Olympus, Stonyfield organic Oikos, Yoplait, Cabot Creamery, and Voskos.

1.5.4 FROZEN YOGURT

Frozen yogurt is generally referred to as the frozen ice milk possessing a typical yogurt flavor. In the United States, frozen yogurt is a blend of 90% ice milk mix and 10% plain yogurt (Chandan and Kilara, 2013). The pH of most popular frozen yogurt is around 6 and it tastes more like ice milk/cream with a hint of yogurt. In some markets, frozen yogurt is made from 100% plain yogurt along with stabilizers, corn syrup solids, and emulsifiers. It usually has a pH 4.5 or below, attained by fermentation with the two yogurt starter bacteria.

As for most other dairy products, different regions have different regulatory requirements. Frozen yogurt in the Netherlands must contain at least 70% yogurt and the pH should be less than 5.0. On the
other hand, Australia requires the yogurt content to be greater than 45% and pH less than 4.5. In the United States, acidic frozen yogurt is not liked by most consumers as the pH of frozen yogurt is usually higher than that in the Netherlands and Australia. Frozen yogurts generally contain high sugar content, and contain stabilizers and/or emulsifiers that help maintain the air bubble structure during freezing. As the production of frozen yogurts resembles closely the processing of ice cream, they are commonly manufactured in ice cream factories.

In recent years, frozen yogurt has become the base for development of other innovative products such as frozen flavored yogurt desserts, frozen yogurt novelties on a stick, or as sandwiches. Probiotic organisms such as *Bifidobacterium* spp. and *L. acidophilus* have been successfully added in frozen yogurt. Frozen yogurt has been used as a carrier of probiotic organisms, enabling the organisms to maintain their viability in low pH and low storage temperature (−29°C). The viability of these probiotic bacteria poses a challenge in Australian frozen yogurts with low pH (4.0–4.5). Furthermore, other means such as ultrafiltered milk have also been used for manufacture of frozen yogurt. Novel cereal-based frozen yogurts have been produced and they were found to possess serum lipid-lowering potential in rats (Ye et al., 2016).

### 1.5.5 SOY YOGURT

Soybeans are a rich and inexpensive source of proteins (36%–38%), and contain less fat (18%–19%) and are low in carbohydrates (28%–30%). Beany flavor and presence of oligosaccharides are two major issues with processing of soymilk. Fermentation of soymilk with *Bifidobacterium* spp. can help overcome the beany flavor in soymilk and the presence of oligosaccharides. The beany flavor of soymilk is attributed to the volatile compounds, *n*-pentanal and *n*-hexanal, that are naturally found in soymilk. The oligosaccharides, mainly stachyose and raffinose, when consumed via soymilk may be responsible for the release of CO$_2$ and methane, which in turn may lead to flatulence during metabolism of oligosaccharides by gut microbes.

Soy yogurt has been manufactured by fermentation of soymilk with *L. bulgaricus* alone. Soy yogurt has also been prepared with fortification of soymilk with cow’s milk or skim milk powder (up to 3%). Fortification with these usually contributes to enhanced flavor, and firm and smooth texture. Bifidobacteria are reported to metabolize the complex sugars stachyose and raffinose, and these sugars in soymilk are reported to stimulate the growth of starter organisms used for yogurt making. Heating the soymilk at 60°C for 15–60 min with added glucose and lactose improved the growth of *L. paracasei* ssp. *paracasei* and *L. helveticus*. Sucrose supplementation in soymilk enhances the growth of *L. acidophilus* in soymilk supplemented with *Streptococcus thermophilus*, and *B. bifidum* also stimulated the growth of both *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* in soymilk.

### 1.5.6 YOGURT BEVERAGES

Yogurt beverages, or drinking yogurt, are prepared from a yogurt mix with reduced milk solids, giving it a low viscosity. The steps for manufacture of yogurt drink are similar to that of yogurt, except for breaking of the coagulum after fermentation, achieved by high speed of agitation. Separation of whey in such products is challenging, and it is necessary to include a stabilizer such as gelatin or carboxymethyl cellulose. Drinking yogurt generally consist of 1.5% fat, 9% milk nonfat solids, up to 8% sugar,
0.5% stabilizer, and 5–15% fruit syrup. Pasteurization or ultra-heat treatment is often involved in the production process to enhance the product’s shelf life.

Aneja et al. (2002) described production details of lassi, a yogurt drink popular in India and Pakistan. This product may be salted along with certain spices or may be sweetened with sugar. To extend the shelf life of Lassi, a process for sterilization and aseptic packaging is also described.

In recent years, oat-based yogurt beverages have been developed with typical sensory features as that of a yogurt-based beverage (Luana et al., 2014). Careful selection of processing and fermentation conditions enabled better nutritional and sensory characteristics of oat-based yogurt beverage, and other yogurt beverages in general.

### 1.5.7 PROBIOTIC YOGURT

It is believed that the two yogurt bacteria, *S. thermophilus* and *L. bulgaricus*, are not natural inhabitants of the intestine and do not survive under acidic conditions and the bile concentrations usually encountered in the gastrointestinal tract. Therefore, the trend has been to make probiotic yogurt by incorporating *L. acidophilus*, *L. casei*, and *Bifidobacterium* spp. as a dietary adjunct in addition to starter bacteria. Wang and Chen (2016) have reported on screening of potential LAB for probiotic potential. Yogurt starter cultures containing the two probiotic bacteria are referred to as “AB” cultures. The recent trend has been to incorporate known probiotics (*L. acidophilus*, *Bifidobacterium* spp.) along with traditional yogurt cultures. Fermented milk with only *L. acidophilus* and/or *Bifidobacterium* could be manufactured, however, the incubation period is long and the quality of the product may be affected when fermenting milk with only AB bacteria as *Bifidobacterium* produces high levels of acetic acid. Thus the normal practice is to make products with both starter culture and probiotic bacteria. The fermentation period with such a starter culture is approximately 4h.

Both *L. acidophilus* and *Bifidobacterium* are difficult to propagate because of their specific nutritional requirements. *Bifidobacterium* spp. is not as acid tolerant as *L. acidophilus* and the growth of *Bifidobacterium* species is significantly retarded below pH 4.0. *Bifidobacterium* is anaerobic. *Bifidobacterium* species are fastidious organisms that require specific growth factors and prefer a low oxidation-reduction potential for growth.

The main species believed to have probiotic characteristics are *L. casei*, *Bifidobacterium* spp., and *L. acidophilus*. Some probiotic yogurt products may contain *L. acidophilus* only, while others may contain *L. acidophilus* and *Bifidobacterium*, or *L. acidophilus*, *Bifidobacterium*, and *L. casei* as probiotic organisms in addition to the two starter bacteria. Thus probiotic yogurts may contain up to five different groups of bacteria.


Actimel, a probiotic supplement beverage by Danone, contains *L. delbrueckii* ssp. *bulgaricus*, *S. thermophilus*, and *L. casei*. Stonyfield Farm, a New Hampshire–based producer, has products on the market that contain six cultures: LB, ST, *L. acidophilus*, *Bifidobacterium*, *L. casei*, and *L. reuteri*.

The most commonly used species in commercial products are *L. acidophilus*, *L. casei*, and *Lactobacillus* GG. *L. GG* is a close relative of *L. casei* subgroup *rhamnosus* (ATCC 53103). Table 1.2 lists fermented milk and yogurt containing dietary lactic acid bacteria and probiotic bacteria.
1.6 CURRENT TRENDS IN YOGURT PRODUCTS IN THE UNITED STATES

1.6.1 WHOLE MILK YOGURT

As a consequence of recent publications showing that consumption of moderate amounts of dietary milk fat is not involved in cardiovascular diseases (German et al., 2009; Westman, 2009; Chowdhury et al., 2014), the industry has responded with a variety of whole milk yogurt products. Because of superior flavor and natural origin, consumers are now attracted to whole milk, full-fat milk products, and butter. Current sales data indicate that nonfat and low-fat dairy products are losing their ground to whole milk products. In the United States, Dannon and Yoplait are now marketing whole milk yogurt, including Greek-style yogurt.

1.6.2 INTERNATIONAL-STYLE YOGURT PRODUCTS

Several new distinctive types of yogurt have been introduced recently in the United States. They offer high protein and natural alternatives to Greek yogurt and conventional yogurt. Some such products are:

### Table 1.2 Fermented Milk and Yogurt Containing Lactic Acid Bacteria and Probiotic Bacteria

<table>
<thead>
<tr>
<th>Name</th>
<th>Culture(s) Used</th>
<th>Country in Which the Product Is Popular</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acidophilus milk</td>
<td><em>Lactobacillus acidophilus</em></td>
<td>Europe, North America</td>
</tr>
<tr>
<td>Acidophilin</td>
<td><em>L. acidophilus, Lactococcus lactis</em> kefir grains</td>
<td>Russia</td>
</tr>
<tr>
<td>Acidophilus paste</td>
<td><em>L. acidophilus</em></td>
<td>Russia, Japan</td>
</tr>
<tr>
<td>Acidophilus yeast milk</td>
<td><em>L. acidophilus</em> and sugar-fermenting yeast</td>
<td>Russia</td>
</tr>
<tr>
<td>ACO-yoghurt</td>
<td><em>Streptococcus thermophilus, Lactobacillus bulgaricus</em>, and <em>L. acidophilus</em></td>
<td>Switzerland</td>
</tr>
<tr>
<td>Actimel joghurt</td>
<td><em>S. thermophilus, Lactobacillus casei</em> Imunitass</td>
<td>Germany</td>
</tr>
<tr>
<td>Bioghurt</td>
<td><em>L. acidophilus, S. thermophilus</em></td>
<td>Germany</td>
</tr>
<tr>
<td>Biogarde</td>
<td><em>S. thermophilus, L. acidophilus</em>, and <em>Bifidobacterium bifidum</em></td>
<td>Germany</td>
</tr>
<tr>
<td>Biolact</td>
<td><em>L. acidophilus</em></td>
<td>Russia</td>
</tr>
<tr>
<td>Biokys</td>
<td><em>B. bifidum</em>, <em>Pediococcus acidilactici</em>, <em>L. acidophilus</em>, <em>L. bulgaricus</em></td>
<td>Czechoslovakia, Europe, Balkan</td>
</tr>
<tr>
<td>Cultura-AB</td>
<td><em>L. acidophilus</em>, <em>B. bifidum</em></td>
<td>Denmark</td>
</tr>
<tr>
<td>Kefir</td>
<td><em>L. casei</em>, <em>L. lactis</em>, <em>L. acidophilus</em>, <em>L. bulgaricus</em>, <em>Kluyveromyces fragilis</em>, <em>Candida kefir</em> (kefir grains)</td>
<td>Middle East, North Africa, Russia</td>
</tr>
<tr>
<td>LC1 or LC1 drink</td>
<td><em>L. acidophilus</em></td>
<td>France, Germany</td>
</tr>
<tr>
<td>Pastolakt</td>
<td><em>L. acidophilus</em></td>
<td>Russia</td>
</tr>
<tr>
<td>Tvorog stolichnaya</td>
<td><em>L. acidophilus</em></td>
<td>Russia, Japan</td>
</tr>
<tr>
<td>Yakult</td>
<td><em>L. casei</em> (Shirota) and <em>L. acidophilus</em></td>
<td>South East Asia</td>
</tr>
</tbody>
</table>

Tarte Asian Yogurt, a Vietnamese yogurt with a lighter texture and less sweet taste as compared to most fruit-flavored yogurts. It also offers unique flavors like green tea and honey as well as mango and coconut. Cooking milk and sugar together to create caramelized color and flavor before culturing makes this product different as compared to traditional yogurt. The product resembles *mishti doi* of India and Bangladesh (Aneja et al., 2002). It has lighter texture. It also contains much higher protein content (13–14 g protein/6 oz serving).

Another product is Icelandic yogurt, called Smari Organics. It is made from organic milk of grass-fed cows and has much higher protein content (17–20 g per 6 oz serving of 6 oz).

An Aussie-style yogurt called Noosa Finest Yogurt is getting popular. The product is made with all-natural ingredients with lower sugar content and offers exotic flavors. Another product is called farm-style Greek yogurt produced by a straining process to give a high protein content (14 g/5.3 oz serving). Among other international-style yogurts are Siggi’s Icelandic yogurt. It is based on skyr, Scandinavian strained fermented milk. It is made from milk of cows not treated with recombinant bovine growth hormone and contains low sugar, fruits, and grains; German quark-based yogurt is also available. Trimona’s Bulgarian-style yogurt is an unstrained yogurt made from grass-fed cow’s milk and is low in sugar.

The international yogurts offer distinct formulation and taste experience. Also, savory yogurt containing vegetable filled yogurt has been introduced.

### 1.6.3 Organic Yogurt

In response to consumer demand, organic yogurt products made from organic-certified milk, organic fruit, and organic cane sugar have been introduced. An example is Liberté yogurt by Yoplait. Stonyfield (Dannon) has a similar product as well. The milk used for the yogurt is from cows not treated with bovine growth hormone. Some products are made from milk derived from cows fed green grass and housed in open areas.

### 1.6.4 Kefir Drinks

Lifeway Foods has been marketing plain and fruit-flavored kefir drinks for many years. They are touted from their health-promoting properties.

### 1.7 Global Trade of Yogurt

Some of the major players operating in the yogurt market include Danone Groupe SA, Yoplait, Ultima Foods Inc., Chobani Inc., Kraft-Heinz Foods Group, Inc., Nestle SA, and Yakult Honsha Co., Ltd., among others. Yogurt is now included as a staple in the diet of people in the United States and in many other countries around the world. The average consumption of yogurt around the world has been steadily increasing.

In 2012, the per capita sales of yogurt (in kilograms) was 21.3 in France, followed by 13.2 in Ireland, 12.4 in Canada, 10.2 in the United Kingdom, 9.9 in Australia, 7.0 in Brazil, 6.7 in the United States, 4.4 in Russia, 3.4 in China, 0.4 in India, and 0.3 in Indonesia.

In the United States, total sales of yogurt in 2015 amounted to 7.7 billion US dollars. In 2010, sales were 6.2 billion dollars. The average per capita consumption of yogurt in 2013 was 14.9 lb (6.5 kg), which was remarkably higher as compared to 2.0 lb (0.9 kg) in 1980. Accordingly, a seven-fold increase has been observed.
In Canada, the consumption of yogurt has been steadily increasing since the 1980s. The consumption increased from 2.69 L per capita in 1986 to 6.77 L per capita in 2005, and steady growth has been observed over the past few decades (Canadian Dairy Information Centre). The yogurt market in Canada is largely dominated by Danone, which has a range of products in the market. The global trade summary for 2015 is shown in Fig. 1.1.

The global trade statistics indicate that total import value of yogurt around the world was almost 2.4 million US dollars in 2015, which is the lowest in the past 5 years. The major yogurt importing countries in 2005 were the United Kingdom and Spain. However, in 2015, Italy was the largest importer of yogurt, importing about 160,000 tons of yogurt, with a total trade value of around 240,000 US dollars. United Kingdom, Spain, Portugal, and Germany were the other leading importers of yogurt in 2015.

**FIGURE 1.1**
Global trade summary.

*Information source: International Trade Centre.*
Geographically, North America is expected to be the major contributor in terms of value followed by Europe in yogurt market. Introduction of new flavors coupled with offering low-fat products may fuel the growth of yogurt market in this region. In developing countries of the Asia Pacific region, China is expected to dominate the market in terms of yogurt consumption followed by India. Moreover, Japan is expected to show substantial growth during the forecast period. Increasing consumption of flavored yogurt especially in countries such as China and Japan is predicted to support the growth of yogurt market across the Asia Pacific region during the forecast period.

In the yogurt export market, the total export value around the world in 2015 was more than 2 million US dollars, with around 1.5 million tons of yogurt being exported worldwide. Germany has been the leading exporter of yogurt for many years, with the trade value of more than 500,000 US dollars in 2015. Germany exported around 500,000 tons of yogurt in 2015, which accounts for more than 30% of the yogurt traded globally. France ranks second in yogurt export, with the trade value of around 300,000 US dollars. Saudi Arabia, Austria, Greece, and Spain are some of the other leading exporters of yogurt around the world. While there has been a decline in the value exported from France, Saudi Arabia has been emerging fast among the yogurt exporters.

The global trend in yogurt production over the past 5 years is shown in Fig. 1.2. As per the latest report by the International Dairy Federation (IDF, 2016), the average yogurt consumption has
increased globally over the past 5 years. More than 10% increase in purchase (or sales in volumes) from 2010 to 2015 was recorded in the United States, Canada, Mexico, Chile, Norway, Sweden, South Africa, Zimbabwe, China, and Japan. Australia, Denmark, and Germany reported 5–10% increase in purchase (or sales in volumes) of yogurt from 2010 to 2015. During the same time period, India, New Zealand, Iceland, United Kingdom, Lithuania, and, Belgium showed 1–5% increase; 5–10% decline was observed in Argentina, France, and Israel, and 1–5% in Spain. The decline in these few countries was attributed mainly to the internal problems of the countries such as poor economy, decline in the currency value, and change in government policies. Overall, the report indicated that yogurt and other dairy foods’ production and consumption would increase globally over the next decade.

In a survey by Nielsen (2012), out of 25,000 participants over 56 countries, it was found that 27% of the participants buy yogurt with acidophilus/cultures due to their health benefits. Standard yogurt makes up 75% of the total market and yogurt drinks make up 25% of the total yogurt market. Yogurt drinks have seen and are expected to see 4.3% growth, and standard yogurt has seen 3.8% growth in the period 2012–17. Top 10 markets by value (in $bn) are China (8.5), United States (8.6), Japan (3.7), Germany (3.1), France (3.1), Netherlands (2.7), Italy (2.3), UK (2.2), Brazil (1.6), and Spain (1.4).

With the increasing awareness about food ingredients and health benefits, yogurt consumers are now focusing on low or no fat, high fiber, low/no sugar, high vitamin, and no additives. When choosing a food or drink, 40% of the consumers prefer flavored yogurt followed by nutrition information (37%), and price (26%). The 20 most popular flavors include strawberry, peach, vanilla, blueberry, raspberry, banana, mango, apple, cherry, pineapple, apricot, chocolate, orange, lemon, pear, honey, passion fruit, coconut, cereal, and blackberry.

Markets for Greek yogurt have surged over the past decade; Chobani has approximately 48% of the market share, followed by Danone (20%), Fage (14%), General Mills (Yoplait) (6%), and other (about 14%). According to a survey, 58% of consumers indicated that they purchase Greek yogurt for its taste, followed by healthiness (45%), high in protein (44%), and texture (39%).

Over 3100 new yogurt products were launched between 2012 and 2014 in Europe alone, followed by North America (1100), Asia Pacific (1050), South and Central America (400), and the Middle East and Africa (100). The top 10 countries in which new yogurt products were launched between 2012 and 2014 included the United States (1000), United Kingdom (600), Germany (500), China (300), Italy (280), Russia (270), Spain (210), France (205), Australia (190), and Brazil (170). The top 10 companies launching new yogurt products include Danone (330), Nestle (90), Valio (75), Marks and Spencer (72), General Mills (60), Walmart (55), Muller (54), Tesco (48), Unilever (46), and Ehrmann (44). Among the new yogurt launches between 2012 and 2014, the top 12 label claims include private label (1300), low fat (800), no artificial color (410), organic (310), no artificial flavor (305), natural (260), high vitamins (55), no preservatives (210), no fat (205), high calcium (200), no gluten (190), and high protein (100). For new yogurt product launches between 2012 and 2014, package types include cup (1300), sleeve (800), bottle (410), box (310), wrapper (300), tub (250), bag (240), resealable (210), carton (205), tray (190), jar (150), and pouch (100). The top 10 countries for the yogurt market from 2013 to 2018 include China ($ million, 13,000), Japan (4250), Australia (1750), and Taiwan (below 1000), Indonesia, Thailand, Korea, New Zealand, India, and Malaysia.

The frozen yogurt industry has seen growth of 74% from 2011 to 2013, an increase from 279 to 485$ (million). Average market growth between 2013 and 2018 is expected to be China (7.5%), Japan (3.5%), Australia (5%), Taiwan (2.5%), Indonesia (4.1%), Thailand (0.5%), Republic of Korea (1.95%),
New Zealand (5%), India (6.4%), Malaysia (9%), Hong Kong (6%), Singapore (5.2%), Philippines (4.2%), and Vietnam (5.7%). Overall, the trends look promising and the global trade and consumption are expected to increase in the coming years.

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**FURTHER READING**

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2.1 INTRODUCTION

The production of yogurt starts with proper selection of raw materials and accurate formulation to produce consistent quality of a liquid mix conforming to a particular type of yogurt under production. In the United States, yogurt is a Grade A product (United States Department of Health and Human Services, 2013). Grade A implies that all dairy components used must come from the US Food and Drug Administration (FDA) supervised Grade A dairy farms and Grade A manufacturing plants, as per regulations put forth in the Pasteurized Milk Ordinance (USDHH, 2013; Frye and Kilara, 2016). All the dairy raw materials should be selected for high bacteriological quality for securing best flavor potential in yogurt. Milk for yogurt production must be free of any inhibitor that might retard the growth of yogurt culture. Milk and other ingredients containing antibiotics are known for production losses in the manufacture of yogurt (Chandan and O’Rell, 2013). One of the two organisms in yogurt culture, *Streptococcus thermophilus*, is particularly sensitive to antibiotics (0.01–0.05 IU/mL of penicillin). Regular testing for antibiotics in milk in the plant laboratory and other measures connected with the use of antimastitis drugs on the farm represent a good system for controlling these residues. In addition to antibiotics, residual disinfectants and sanitizing chemicals may inhibit the growth of starter cultures. Chlorine compounds such as hypochlorites and iodophors may slightly inhibit starter cultures at a level of 6–10 μg/mL of milk. Also, quaternary ammonia compounds inhibit lactic acid bacteria at concentrations as low as 0.1–1.0 μg/mL, depending on particular strain sensitivity. Accordingly, normal precautions regarding the use of sanitation chemicals on the farm should greatly reduce the chance of obtaining residual levels affecting yogurt cultures.

2.2 COMPOSITION OF YOGURT MIX FOR YOGURT PROCESSING

Yogurt mix composition regarding milk fat and milk solids-not-fat is generally standardized from whole, partially defatted milk, condensed skim milk, cream, and/or nonfat dry milk by the use of appropriate software programs. The chemical composition of dry dairy ingredients used in yogurt manufacture is given in Table 2.1.
The FDA Code of Federal Regulations (US Department of Health & Human Services, 2015; Frye and Kilara, 2016) standard of identity for yogurt, low-fat, and nonfat yogurt calls for a minimum of 8.25% nonfat milk solids (SNF) in the fermented mix “before the addition of bulky flavors.” In a typical nonfat, low-fat, or full-fat yogurt formulation, the total milk serum solids (or solids-not-fat) content of yogurt mix ranges from 8.25% to 12.00%, depending on the choice of stabilization. The serum solids associated with the fluid portion of milk are usually 8.8%–9.0%. The addition of sugar, stabilizers, or other dry ingredients will dilute the total SNF level. Additional nonfat dry milk (NFDM) solids are added to the yogurt mix to achieve the 8.25% minimum SNF requirement, to build up the total solids and to increase the protein content. Generally, the addition of 1%–3% NFDM solids raises the protein level sufficiently so that with the proper heat treatment, there is an increase in bound water leading to improved firmness and consistency of the coagulum. Another benefit is the control of wheying off or syneresis on the surface of yogurt. For use in yogurt, only low-heat NFDM is used.

In the manufacture of flavored yogurt, it is usually desirable to add a sweetening agent to the yogurt base. The level of sweetness in the yogurt mix will depend on the °Brix of the fruit or flavoring ingredient and the desired level of sweetness in the finished product. Most fruit-flavored yogurts contain approximately 10%–13% sugar equivalent, while flavored yogurts (vanilla, lemon, coffee, etc.) contain 8%–10% sucrose. The sweetener most commonly used in the industry is sucrose in either liquid (65%–67% total solids) or granulated form. The total amount of sugar solids in yogurt mix prior to fermentation should not exceed 10%–11% because of the inhibitory effect on the traditional yogurt culture. The addition of the sugar generally occurs before pasteurization. To produce “light” or reduced-sugar/low-calorie yogurts, approved nonnutritive high-intensity sweeteners are used.

Table 2.1 Typical Chemical Composition of Dairy Ingredients Used in Formulation of a Yogurt Mix

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Total solids (%)</th>
<th>Fat (%)</th>
<th>Protein (%)</th>
<th>Lactose (%)</th>
<th>Ash (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole milk</td>
<td>12.6</td>
<td>3.5</td>
<td>3.5</td>
<td>4.9</td>
<td>0.7</td>
</tr>
<tr>
<td>Skim milk</td>
<td>9.5</td>
<td>0.1</td>
<td>3.6</td>
<td>5.1</td>
<td>0.7</td>
</tr>
<tr>
<td>Whipping cream</td>
<td>42.7</td>
<td>36.8</td>
<td>2.2</td>
<td>3.2</td>
<td>0.5</td>
</tr>
<tr>
<td>Condensed skim milk</td>
<td>40</td>
<td>0.4</td>
<td>39.6</td>
<td>10.8</td>
<td>2.22</td>
</tr>
<tr>
<td>Nonfat dry milk</td>
<td>96.5</td>
<td>0.8</td>
<td>35.9</td>
<td>52.3</td>
<td>8.0</td>
</tr>
<tr>
<td>WPC-34</td>
<td>96.5</td>
<td>4.0</td>
<td>34.5</td>
<td>51.0</td>
<td>7.0</td>
</tr>
<tr>
<td>WPC-50</td>
<td>96.5</td>
<td>4.0</td>
<td>50.5</td>
<td>36.0</td>
<td>6.0</td>
</tr>
<tr>
<td>WPC-80</td>
<td>96.5</td>
<td>6.0</td>
<td>80.5</td>
<td>5.0</td>
<td>5.0</td>
</tr>
<tr>
<td>Whey protein isolate</td>
<td>96.5</td>
<td>0.5</td>
<td>93.0</td>
<td>1.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Fluid UF milk</td>
<td>25–30</td>
<td>11–14</td>
<td>10–12</td>
<td>&lt;5</td>
<td>&gt;2.5</td>
</tr>
<tr>
<td>Fluid UF skim milk</td>
<td>15–20</td>
<td>&lt;0.5</td>
<td>10–12</td>
<td>&lt;5</td>
<td>&gt;2.5</td>
</tr>
<tr>
<td>Fluid UF skim milk, with diafiltration</td>
<td>18–20</td>
<td>&lt;0.5</td>
<td>16–17</td>
<td>&lt;1</td>
<td>&gt;1.5</td>
</tr>
</tbody>
</table>

aWhey protein concentrate.

Stabilizers are hydrocolloids of plant and animal origin. The primary purpose of adding stabilizers in yogurt is to improve consistency and build viscosity, to minimize whey separation and bind free water, and to maintain the gel structure after pumping, mixing, and cooling. The stabilizer increases shelf life of the product and provides a reasonable degree of uniformity from batch to batch. Stabilizers function through their ability to form gel structures in water, thereby leaving less free water for syneresis. In addition, some stabilizers complex with casein providing added body and further protection against syneresis.

Preferably, the incorporation of the stabilizer should take place using a high shear-type blender that has strong agitation resulting in complete dispersion and a uniform suspension. There are many stabilizers and their combinations available in the industry for use in yogurt.

Gelatin of Bloom strength of 225 or 250 is commonly used. The gelatin level should be geared to the consistency standards for yogurt. Amounts above 0.35% tend to give yogurt of relatively high milk solids a curdy and lumpy appearance upon stirring. Gelatin tends to degrade during processing at ultrahigh temperatures, and its activity is temperature dependent. The yogurt gel developed by gelatin is considerably weakened by a rise in temperature. Generally, combinations of modified starch-gelatin or gelatin-pectin are used. The stabilizer combination ratios as well as the final concentration (generally 0.50%–2.00%) in the product are carefully controlled to get desirable effects.

The stabilizers generally used in yogurt are shown in Table 2.2.

<table>
<thead>
<tr>
<th>Stabilizer</th>
<th>Concentration in Yogurt Mix (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pectin (low methoxy for yogurt)</td>
<td>0.08–0.20</td>
</tr>
<tr>
<td>Pectin (high methoxy for yogurt beverages)</td>
<td>0.30–0.50</td>
</tr>
<tr>
<td>Starch, modified (tapioca/corn)</td>
<td>0.8–2.0</td>
</tr>
<tr>
<td>Gelatin (225/250 Bloom)</td>
<td>0.1–0.5</td>
</tr>
<tr>
<td>Locust bean gum</td>
<td>0.3–0.5</td>
</tr>
<tr>
<td>Xanthan gum</td>
<td>0.01–0.05</td>
</tr>
</tbody>
</table>


Table 2.2 Stabilizers and Their Concentrations for Use in Yogurt and Yogurt Drinks

Modified corn/tapioca starch suitable for use at low pH is commonly used in yogurt formulation. For instance, stabilized and medium cross-linked waxy maize starch (hydroxypropyl distarch phosphate) is a viscosity generator and a stabilizer. It has a bland flavor, gives clear paste, smooth short texture, and can withstand severe processing conditions of low pH, high heat, and extreme shear.

Pectins are commonly used alone or in combination with other hydrocolloids to stabilize stirred and set yogurt. Low-methoxy pectin is the preferred type for (refrigerated) cup yogurt. Very small amounts (0.07%–0.15%) modify the consistency of the yogurt making it stiffer and preventing any syneresis that might arise during handling, transportation, and distribution. High-methoxy pectin is preferred to ensure stability and control viscosity in acidified milk drinks.

Table 2.3 shows typical formulation of some popular yogurt products. The formulation of a given yogurt mix is standardized as per the regulatory and marketing requirements.
2.3 MANUFACTURING STEPS FOR MANUFACTURE OF MAJOR TYPES OF YOGURT

Readers interested in more details on manufacture of yogurt and related products may refer to publications on this subject (O’Rell and Chandan, 2013b; Chandan, 2014).

In general, the following steps are needed in yogurt production.

1. **Blending**: The liquid ingredients are measured into processing vats followed by the addition of solid ingredients. It is necessary to homogeneously disperse and dissolve the dry ingredients in the liquid phase by the use of high-shear mechanical blenders.

2. **Pasteurization and heat treatment**: Generally, pasteurization of milk is carried out with the purpose of killing all the pathogenic microorganisms, significantly reducing the majority of other organisms present, and inactivating the inherent enzymes of milk. A high-temperature short-time heat exchanger with long holding tube is generally used in the yogurt industry.

   In the US yogurt processing industry, the FDA regulations require plant operators to install legal pasteurization equipment, although the heat treatment of yogurt mix uses higher temperatures with a longer holding time than legal milk pasteurization. The heat treatment denatures whey proteins and creates optimum conditions for the growth of yogurt culture. Extensive denaturation of whey proteins (80%-85%) increases their water binding capacity, which improves the consistency and viscosity of yogurt and helps to prevent free whey separation (syneresis).

3. **Homogenization**: This process of mechanically breaking milk fat globules into smaller sizes helps in more uniform dispersion of stabilizers in yogurt mix.

   It also prevents rise of cream layer in yogurt vessels and cups. However, in some Greek-style yogurts and natural whole-milk yogurts, no homogenization is carried out because a cream layer on the top is desired.

   In the manufacture of “natural” yogurt with minimum stabilization (milk solids-not-fat 11%-13%), a high pressure of homogenization of approximately 23–28 MPa/6 MPa

<table>
<thead>
<tr>
<th>Yogurt Style</th>
<th>Milk Fat (%)</th>
<th>Milk Solids-Not-Fat (%)</th>
<th>Sugar Solids (%)</th>
<th>Stabilizer (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plain: whole milk</td>
<td>3.25–3.50</td>
<td>11.0–12.0</td>
<td>0</td>
<td>0–1.0</td>
</tr>
<tr>
<td>Plain: low fat</td>
<td>1.0</td>
<td>14.2</td>
<td>0</td>
<td>0–0.75</td>
</tr>
<tr>
<td>Plain: nonfat</td>
<td>0.3–0.5</td>
<td>14.0–15.0</td>
<td>0</td>
<td>0–0.5</td>
</tr>
<tr>
<td>Fruit blended: whole milk</td>
<td>3.25–3.50</td>
<td>10.5–11.0</td>
<td>6.0–10.0</td>
<td>0.4–0.6</td>
</tr>
<tr>
<td>Fruit blended: low fat</td>
<td>&gt;0.5–&lt;2.0</td>
<td>10.5–12.0</td>
<td>6.0–10.0</td>
<td>0.3–0.4</td>
</tr>
<tr>
<td>Fruit-blended: nonfat</td>
<td>0.3–0.5</td>
<td>11.0–12.0</td>
<td>6.0–10.0</td>
<td>0.3–1.2</td>
</tr>
<tr>
<td>Yogurt: drink/smoothie</td>
<td>&lt;2.0</td>
<td>8.0–9.5</td>
<td>8.0–12.0</td>
<td>0.01–0.5</td>
</tr>
</tbody>
</table>

Table 2.3 Typical Composition of Some Popular Yogurt Products

(2000–2500/500 psi), double stage, or 23–28 MPa (2000–2500 psi), single stage, is used to improve consistency and help prevent whey separation.

4. **Preparation of bulk starter:** Fig. 2.1 shows an outline for production of yogurt starter. The starter tank valves and pipes and hatch with gasket are assembled and sterilized with live steam at low pressure (3–5 lbs). The bottom valve is kept open for the condensate to drain. The tank is steamed for 30 min after the surface temperature in the tank has reached $\sim 99^\circ C$ ($210^\circ F$). At this point, the steam is turned off and the bottom valve is closed (Chandan and Nauth, 2012; Chandan, 2016b).

Skim milk with total solids raised to 10%–12% is either pumped cold and heated to 90°C (194°F) and held for 60 min or the starter mix is heated to 90°C (194°F) in a plate heat exchanger and then held at that temperature in the tank for 60 min. Some plants prefer to use reconstituted nonfat dry milk at 10%–12% total solids for their bulk starter. The mix is cooled to 43°C (109°F). For 500 gallons, a can (350 mL) of frozen culture concentrate ($10^{10}$ cfu/g) is thawed in 5 gallons of tepid water containing 100 ppm chlorine. The tank is inoculated with thawed starter concentrate, followed by stirring the mixture for 5 min. The agitator is turned off and incubation of inoculated mix is continued quiescently for 6–8 h to reach 0.9% titratable acidity. The starter is cooled to 5°C (41°F) using slow agitation. Then agitation is turned off. The agitation is resumed for a few minutes before the starter is to be pumped. For a healthy culture, it may take 8–10 h to reach 0.9% acidity. Starter has $2–5 \times 10^8$ cfu/mL.

5. **Addition of starter and fermentation:** The homogenized yogurt mix is tempered to fermentation temperature (42°C/111°F) in a plate heat exchanger and pumped to fermentation tanks. The mix is then inoculated with yogurt starter (at 1%, v/v) and allowed to incubate quiescently until the pH reaches 4.6. During yogurt fermentation, lactic acid is produced as a result of bacterial growth. Concomitantly, typical yogurt flavor is generated and the mix acquires a coagulated, thick set consistency.
6. **Cooling**: The objective of cooling of fermented mass is to restrict the growth of yogurt culture and its enzyme activity as quickly as possible and maintain the desired pH, body, and texture. Generally, cooling in yogurt plants should take place at pH 4.5–4.6. Cooling with agitation at pH 4.7 or above can result in a grainy body and undesirable texture in the finished yogurt. The rate of cooling should be steady but not too fast. Cooling too rapidly can bring unfavorable changes in the structure of the coagulum contributing to whey separation in the finished yogurt. The method of cooling depends on the style of yogurt that is being produced. It is desirable to reach a temperature of 18–20°C within 1 h to quickly stop further culture growth. Cup-incubated yogurt is cooled in the retail containers using a blast of cold circulated air in a cooling chamber/cell or a blast cold tunnel. High-velocity air creates simulated windchill conditions. Vat-incubated yogurt is cooled using a special plate cooler, a multitube cooler, or in some cases in a processing vat with the circulation of refrigeration water in its jacket wall and agitation of the coagulum in the vat. When cooling in the vat, it is better to use a narrow high tank with swept surface agitation for quick cooling of the gel. Wide and high processing tanks with a propeller-type agitator are unfavorable for cooling. Many plants pump their cooled fermented base through a back pressure valve, a perforated stainless steel disk, a stainless steel mesh screen or a “sour cream” cone in the line to ensure a smooth texture in the fermented mass.

In the vat-incubated yogurt, the temperature of filling varies according to the type of stabilizers used. Generally, it is desirable to cool the yogurt to 7–13°C (45–55°F).

7. **Stirring**: Stirring should not be too rigorous or too long. This is especially important in the manufacture of natural yogurts; however, it should also be considered in stabilized yogurt since excessive stirring may break down some of the stabilization and change the level of stabilizer needed to obtain desirable body. To obtain a homogeneous gel, it is preferable to use a higher rate of stirring initially, reducing the rate of agitation as the temperature drops below 30°C (86°F).

8. **Pumping**: Pumps are needed to transport stirred yogurt from fermentation tanks through pipes and possibly a plate cooler to the filling machine. They operate with different pressures, depending on the design. However, for this application only positive drive pumps should be used. This ensures a positive displacement of the gel without impairing its structure. Centrifugal pumps should not be used because the high centrifugal force produced by the rotary propeller forces the product to leave the pump with high speed and high pressure, which damages the gel consistency resulting in a weaker body.

2.4 **GENERAL MANUFACTURING PROCEDURES**

2.4.1 **PLAIN YOGURT**

Plain yogurt may be full fat, low fat, or nonfat. It is made either by cup incubation or by vat incubation. The total solids range from 12.0% to 14.0%. The steps involved in the manufacturing of set-type plain yogurt are shown in Fig. 2.2.

Plain yogurt normally contains no added sugar or flavors to provide the consumer natural yogurt flavor for consumption or as an option of flavoring with other food materials of the consumer’s choice. In addition, it may be used for cooking or for salad preparation with fresh fruits or grated vegetables.
2.4.2 FRUIT-FLAVORED YOGURT

Fig. 2.3 depicts an outline for the manufacture of blended Swiss-style yogurt. For the production of blended/Swiss style, the fermented yogurt base is mixed with various fruit preparations. The fruit incorporation is conveniently effected by the use of a fruit feeder or metering pump at a 10%–20% level followed by a static in-line mixer to assure homogenous blending of the fruit with the yogurt base. Another choice is in-line injection of fruit. Prior to flavoring, the texture of stirred yogurt can be made smoother by pumping it through a back pressure valve or a stainless steel screen or mesh.
CHAPTER 2  AN OVERVIEW OF YOGURT PRODUCTION AND COMPOSITION

**FIGURE 2.3**
An outline for the manufacture of fruit-flavored (blended) yogurt.
A common stabilizer blend used for blended yogurt consists of a combination of modified food starch (0.6%–1.5%) and gelatin of 225–250 Bloom (0.25%–0.40%). It produces a creamy, firm yogurt that is resistant to wheying off and stirs out smooth and free of lumps. If a “natural” approach is desired, a gelatin-pectin stabilizer or agar-pectin stabilizer can be used.

Typical flavorings for stirred-style yogurt are as follows:

1. Fruit preparations used at a 10%–18% level:
   a. °Brix: 45–64
   b. % Fruit: 15–35
   c. Sweetener: sugar and/or corn sweeteners
   d. Stabilizer: pectin/modified food starch

2. Flavored syrup or flavor concentrate and/or fruit juice
   a. No visible fruit
   b. Lower calorie versus fruited yogurt
   c. 8%–9% sugar versus 10%–12%

3. Combination of fruit preparation and flavored syrup or flavor concentrate
   a. Economy version
   b. 6%–10% fruit (40–50°Brix)
   c. 2%–4% flavoring

Details of the fruit preparation are given elsewhere (O’Reell and Chandan, 2013a).

Light yogurt is usually made without the addition of sugar. High-intensity sweeteners are used to replace the sugar in the formulation. The synthetic sweetener is added either in the fruit preparation or flavoring or directly to the yogurt base. Light yogurts are stirred style that uses a special fruit preparation characterized by 10–12°Brix, 20%–45% fruit content and is designed for use at 10%–18% level.

Custard style is also a fruit-flavored yogurt. It is a reduced-fat yogurt containing enough starch to create a very firm consistency of yogurt. Furthermore, it contains no fruit chunks and is preferred mostly by children. It is a cup-fermented product. Other children-directed yogurts contain bright colors and are sweeter than regular yogurt. Some products are packaged in a cup to produce multiple-colored vertically deposited layers during packaging. Other yogurts for children are packaged in plastic tubes.

Cup-incubated sundae-style yogurt is produced by incubation of seeded yogurt mix in a cup. In a typical traditional 6 oz cup of sundae-style yogurt, 39 g (1.4 oz) of special fruit preparation is layered at the bottom followed by 131 g (4.6 oz) of inoculated yogurt mix on the top. After the containers are sealed, incubation and setting of the yogurt takes place in the individual cup. When a desirable pH of 4.5–4.6 is attained, the cups are placed in refrigerated rooms, or blast cooling tunnels or cells for rapid cooling. For consumption, the fruit and yogurt layers are mixed by the consumer.

Vat-incubated sundae-style and parfait yogurt is produced by first incubating the mix in the vat and then pumping into the cup with fruit preparation on the bottom. Some parfait yogurts use a blended vanilla-flavored yogurt on top of the fruit layer. A stabilizer is almost always added to the yogurt base.

Another type of commercial product is vanilla-flavored yogurt. In yogurt production it is most typical to use vanilla extracts from 1× (1-fold) to 3× (3-fold). For a 2× vanilla extract a typical usage rate is 0.45%–0.60%. For economic reasons, some manufacturers might choose to blend a vanilla flavor or
vanillin with pure vanilla extract to lower the cost. This would also impact the product label. Some manufacturers prefer to obtain vanilla in processed syrup (vanilla extract in liquid sugar syrup) from a typical fruit preparation supplier. With this syrup, it is possible to flavor both vanilla and fruited yogurts using one plain yogurt base. Whether vanilla yogurt is produced using pure vanilla extract or in a 50–60°Brix syrup, the finished yogurt usually targets a finished sugar content of 8%–9%. This sugar level gives the balance of sweetness and acidity in most yogurts to deliver a well-rounded vanilla flavor for the consumer.

*Organic yogurt* must be produced and processed in accordance with the National Organic Program (NOP) standards as defined by the FDA in 7 CFR Part 205. Under the NOP standards, food products meeting the requirements for “100% organic” and “organic” may display these terms and may use the United States Department of Agriculture (USDA) organic seal. Food products labeled as “100% organic” must contain (by weight, excluding water and salt) only 100% organically produced ingredients including any processing aids. This product category is primarily found in produce, meat, or minimally processed foods. Manufacturers of multiingredient foods, such as yogurt, strive to achieve the organic label. Just the required use of yogurt cultures in the production of yogurt would exclude it from achieving the “100% organic” label because the yogurt cultures are considered nonagricultural but allowed under 205.605(a) of the regulation. To achieve the organic label, yogurt, as well as all products labeled “organic,” must consist of at least 95% organically produced ingredients (by weight, excluding water and salt) and any remaining product ingredients must be organic compliant; that is, consist of nonagricultural substances approved on the National List (FDA, 7 CFR 205.605) or nonorganically produced agricultural products that are not commercially available in organic form (7 CFR 205.606). Any yogurt, or other product, labeled as organic must identify each organically produced ingredient in the ingredient statement on the information panel. The regulations also prohibit the use of genetic engineering, ionizing radiation, and sewage sludge in organic production and handling.

A yogurt manufacturer interested in producing organic products must be certified. Certification standards under the NOP regulations establish the requirements that organic production (crops and livestock) and handling (processing) operations must meet the standards necessary to be certified by a USDA-accredited certifying agent. The information that an applicant must submit to the certifying agent includes the applicant’s organic system plan. The organic system plan describes (among other things) practices and substances used in processing, record-keeping procedures, practices to prevent commingling of organic and nonorganic products, and on-site inspections (O’Rell and Chandan, 2013a,b).

There are many available organic ingredients that can be used in the production of a certified organic yogurt, low-fat yogurt, or nonfat yogurt.

Yogurt drinks/smoothies are designed to be consumed as a beverage or shake. They consist of (1) refreshing low–milk solids drink or (2) a health-promoting yogurt drink supplemented with prebiotics, probiotics, vitamins, and minerals. To be labeled as a yogurt drink, the white mass (yogurt component) of the drink/beverage must conform to the FDA standard of identity that calls for >8.25% milk solids-not-fat and fat level to satisfy nonfat yogurt (<0.50%), low-fat yogurt (2.00%), or yogurt (>3.25%) label, prior to the addition of flavoring ingredients. After the addition of fruit and flavors, the yogurt does not have to meet these standards.

Typically, commercial drinkable yogurt is a low-fat (<2.0% fat) drink containing 8.0%–9.5% milk solids-not-fat and 8%–12% sugar. Its pH varies from 4.0 to 4.5. Reduced-calorie drinks are made with high-intensity sweeteners replacing all the sugar. Yogurt drinks generally contain fruit juices or purees,
although in some markets they may contain sugar only with or without fruit flavors. The fruit content is generally in the range of 8%–15%. In some markets the fruit juice range may be as high as 30%–49%.

Yogurt drinkables and smoothies are of two types: regular and those fortified with prebiotics, probiotics, minerals, and vitamins. The prebiotic, fructooligosaccharides or inulin, along with synergistic probiotic cultures of *Lactobacillus casei*, *Lactobacillus reuteri*, and *Bifidobacterium* is present in addition to yogurt culture. A mixture of hydrocolloids in the range of 0.01%–0.50% is usually employed to stabilize the product from separation. High-methoxy pectin is especially functional in imparting the required viscosity and protein interaction to prevent separation. Another important processing step is low-pressure homogenization at 6 MPa (500 psi) of fermented base to create small casein particles that interact with pectin to stop aggregation of the protein, thereby creating a stable suspension. The production method for drinkable is similar to that of blended/Swiss-style yogurt. The mix contains milk solids, sugar, stabilizers, and optional ingredients consisting of mineral-vitamin supplement, fructooligosaccharide. This blended mix is heat-treated at 85°C for 30 min or 95°C for 10 min to create conditions favorable for culture growth and for viscosity generation. The mix is then cooled to 39–41°C, inoculated with 1%–2% of yogurt starter and optional probiotic cultures, and incubated in quiescent state until a pH of 4.3–4.4 is achieved. The curd is broken while cooling to 18–19°C. The next step is distinctly different for yogurt drinks. Certain processes require addition of pasteurized solution of high-methoxy pectin to achieve 0.3% pectin level in the yogurt drink. The cooled fermented mass is homogenized at low pressure (6 MPa, 500 psi, single stage) to convert casein to low particle size and facilitate interaction with pectin to obtain desired low viscosity and to render stable suspension. At this point, fruit puree and flavoring or syrup may be incorporated. Typical flavorings for yogurt smoothies consist of flavored syrups or flavor concentrate and/or fruit juices. After proper blending, the drink is ready for packaging in paper cartons or bottles. Individual serving bottles are commonly used for yogurt drinkables. On route to the bottle filler, it is desirable to cool the yogurt drink to 5°C by passing it through a plate cooler. Prior to filling the bottles, they are unscrambled and air-blown. They are then turned upside down, rinsed, sterilized, and filled with required weight, followed by sealing with aluminum foil and application of a cap. The finished product is checked for pH, viscosity, and color at regular intervals off the filler. After coding and shrink-wrapping, they are packed in cases and mechanically moved to a cold room. They are then placed on pellets, shrink-wrapped, and transferred to the cooler before being shipped out of the plant.

Yogurt whip/mousse has unique eating quality in that it has a fluffy, light texture and mouth-feel. It adds variety and a new taste sensation to the product portfolio. The foaming of the mix takes place during processing. Compared to stirred-type yogurt, the mix for whipped yogurt contains more sugar and stabilizers. Gelatin is an essential ingredient of whipped yogurt. Low-to-regular fat mix whips better than the nonfat mix. The stability of the foam is facilitated by the use of suitable emulsifiers and stabilizers in the mix. An emulsifier aids in foam formation while stabilizer is responsible for viscosity, mouth feel, and stability of the foam and emulsion structure. The bubbles formed are prevented from collapsing by the action of stabilizer-emulsifier during the shelf life of the product. High altitude can affect the stability of the foam as well. Generally, a stabilizer system includes starch, gelatin, carrageenan, guar gum, xanthan gum, and locust bean gum. The emulsifiers include mono- and diglycerides, especially the lactylated type. The stabilizer-emulsifier blend is directly incorporated in the yogurt mix prior to heat treatment.
After fermentation, the mix is whipped using inert gas like nitrogen to increase the volume of the mix by 50%. Thus, the whipped yogurt has an overrun of 50%. Thus, a 6-ounce cup will hold 4 oz of yogurt whip. During whipping, the high turbulence in the equipment results in fine gas bubbles dispersed in the aqueous phase. The mixing head of the aeration machine disintegrates large gas bubbles into finer bubbles forming desirable foam structure. The emulsifiers are surface active agents. By reducing surface tension, they facilitate bubble formation, while the stabilizers enhance viscosity and form a coating around the bubbles to give them strength and capacity to resist from collapse. The foam matrix consisting of fat globules, gas bubbles, and aqueous phase containing soluble and insoluble components of the mix is formed at low whipping temperature.

Greek-style or strained yogurt constitutes approximately 50% of the total yogurt market. Greek yogurt has traditionally been made from whole milk supplemented with cream to standardize the fat level to 7%. After the fermentation is complete, yogurt is concentrated overnight by straining through a cheesecloth at 4°C. Due to the drainage of whey, the total solids increase from 14% to 21%–23%. The concentration step results in a remarkably thick viscous body. The fat content of this type of yogurt rises to approximately 10%. The high-fat content imparts very creamy flavor and moderates the acid flavor. The fermented protein also concentrates and contributes to smooth texture. The traditional method is labor intensive and lacks sanitation conditions for obtaining desirable shelf life of the product.

The modern processing procedures for Greek yogurt involve whey removal by mechanical procedures. In contrast with regular yogurt production, Greek yogurt manufacture results in large volumes of acid whey as a byproduct. Consequently, Greek yogurt plants must be equipped to handle processing of acid whey or arrange for its sale to whey processors. Acid whey can be dried and sold as an ingredient in food industry. Other plants have invested in its conversion to fuel gases to supplement energy use at the plant. Other options include fractionation of its major components to add value to the by-product.

Drainage of the whey is accomplished by passage of the fermented milk through quarg/centrifugal separators or by the application of ultrafiltration techniques.

In the centrifugal process, the fermented milk is concentrated in a spray nozzle centrifuge, wherein the product enters from the top and goes to the bottom of the bowl via a distributor and then enters the rising channels in the disk stack, where it is separated into curd and whey (Kilara and Chandan, 2013). The separated curds are fed to nozzles through a segmented insert and then discharged out of the separator into a collection bowl and onto a product collector. The whey is discharged through a centripetal pump located at the top of the separator bowl. Assuming that the total solids of the product discharged is 18%, separators with capacities from 1000 to 3000 kg/h (2200–6600 lb/h) are available from suppliers. The described configuration leads to long holding times and little product loss. The product hopper is equipped with sensors to regulate the product pump at different levels. Product output is determined by the diameter of the nozzles. Different nozzle diameters combined with the size of the holes in the plates are used to process various products on the same separator. The concentrated product is then mixed, homogenized, and further processed. Further processing consists of adding colors, flavors, and inclusions prior to packaging and distribution. Thus, skim milk with approximately 9% total solids is concentrated to Greek-style yogurt with 18% total solids prior to the addition of flavors, colors, and inclusions.
Another method of concentrating solids uses membrane filtration. Ultrafiltration is the process in which macromolecules are concentrated. The major macromolecules in milk are fats and proteins. This technique utilizes a cross-flow membrane in which the feed solution is forced through the membrane under pressure. The solution flows over the membrane and solids are retained (retentate) while the removed materials are present in the permeate. The filter modules are available in various geometries. Spiral wound are the most common but others available are plate and frame, tubular, and hollow fiber. Tubular filters can be made out of ceramics or polymers. Membrane operations can be batch or continuous. In dairy plants, continuous processes are more desirable. Process temperatures are maintained at around 50°C to minimize microbial growth and to improve membrane flux.

The use of membrane processing in the cultured dairy products area is restricted to concentration of skim milk for fat-free yogurt manufacture. Some of the lactose and minerals are removed from skim milk thereby increasing the protein content. This process can concentrate skim milk (9% solids) to 12% solids. There is still enough lactose in the retentate to facilitate fermentation. A higher protein content in the concentrated milk results in a firmer acid gel in yogurt. Membrane processes are extensively used in the manufacture of whey protein and milk protein concentrates and isolates.

Use of membrane processing in Greek-style yogurt consists of (1) concentrating the milk prior to yogurt manufacture or (2) concentrating yogurt to remove whey solids.

Ingredient additions take place after homogenization and prior to filling. Mainly, these ingredients are colors, flavors, and inclusion such as fruit pieces, nuts, and variegates.

When skim milk is concentrated prior to culturing, whey proteins are retained along with casein. The minerals associated with casein micelles are not changed when milk is ultrafiltered at its native pH of 6.7. The concentration of these minerals increases in the same proportions as that of protein. Increased mineral content results in increased buffering capacity of the retentate. Consequently, when the retentate is fermented by the yogurt cultures to the same end point pH as regular milk, the product will be more acidic. During acidification more colloidal calcium is solubilized from the micelle and migrates to the aqueous phase leading to alterations in the aggregation of casein.

When acidified milk is ultrafiltered, the soluble solids traverse the membrane and into the permeate thus lowering their concentration. Ultrafiltration of yogurt not only concentrates solids but also results in products with less acidity because lactic acid passes into the permeate. The viscosity of retentate, whether concentrated prior to or after acidification, increases with increasing protein concentration. Heating the retentate denatures whey protein thus resulting in increased water-holding capacity and therefore yield of product.

Ultrafiltration of yogurt postfermentation removes lactic acid to some extent, and the end product will taste less acidic than Greek-style yogurts produced by other methods. The body, texture, and appearance of this product are better than yogurt made from other processes. Ultrafiltration of skim milk results in a 2-fold volume concentration prior to heat treatment, followed by fermentation with yogurt culture. The resulting concentrated yogurt is subsequently packaged.

In the United States, two methods are primarily used to produce Greek yogurt. One method follows the traditional process of using a quark (or quarg)-type separator to strain the liquid whey after the yogurt is set through fermentation. In this case the yogurt passes through the separator at incubation temperatures, flavorings may be added, and the product is filled and subsequently cooled in the container using blast cooling. Another method is to start with fluid milk or skim milk fortified with milk protein concentrate (MPC) or a
functional blend of MPC and whey protein concentrate (WPC). Proper selection and processing of the protein concentrates is critical to obtain a finished product with a clean flavor and no graininess or chalky mouthfeel. The protein-fortified milk method allows Greek yogurt to be produced on existing yogurt processing equipment avoiding the capital investment of straining and blast cooling equipment. It also eliminates the need to treat the waste effluent stream that comes from straining the yogurt. Still another less-used method is to ultrafiltrate the milk at the beginning of the process to concentrate the protein before fermentation. This is a more challenging process to obtain the optimum smooth texture.

*Frozen yogurt:* Currently, no federal standards have been approved for frozen yogurt. The product may be defined as a food prepared by freezing while stirring a blend of pasteurized nonfat or low-fat ice cream mix and yogurt (O’Reill and Chandan, 2013b). Yogurt used for blending with nonfat or low-fat ice cream mix must comply with the federal and state compositional standards for yogurt. Yogurt must be cultured with yogurt culture containing *Lactobacillus bulgaricus* and *S. thermophilus* to titratable acidity of minimum of 0.85%. In general, frozen yogurt mix obtained by blending yogurt and low-fat/nonfat ice cream has a pH of 6.0 or titratable acidity of 0.30%. Thus, the industry standards require minimum titratable acidity of 0.30%, with a contribution of approximately 0.15% as a consequence of fermentation by yogurt bacteria. Most manufacturers use 10% of yogurt in their formulations. As a consequence, frozen yogurt tastes very similar to low-fat/nonfat ice cream, with a hint of yogurt flavor at the end. This flavor attribute is preferred by the consumer in that the perceived health attributes of yogurt bacteria are available along with the popular taste of low-fat/nonfat ice cream. Frozen yogurt is labeled according to the fat content of standard serving size (4 fl oz) used in the ice cream industry. Accordingly, the product containing >3 g of fat per 4 fl oz is labeled as frozen yogurt; the product containing 0.5–3.0 g per 4 fl oz is low-fat frozen yogurt, and the product with <0.5 g fat is labeled nonfat frozen yogurt.

A typical formulation of nonfat frozen yogurt is given in Table 2.4. The table shows a mix composed of 10% sweetened nonfat plain yogurt and 90% nonfat ice cream mix. If a lower pH (<6.0)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Plain Nonfat Yogurt, 10%</th>
<th>Nonfat Ice Cream Mix, 90%</th>
<th>Frozen Yogurt Mix</th>
</tr>
</thead>
<tbody>
<tr>
<td>Milk fat (%)</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
</tr>
<tr>
<td>Milk solids-not-fat (%)</td>
<td>10.96</td>
<td>10.02</td>
<td>10.11</td>
</tr>
<tr>
<td>Whey protein concentrate (%)</td>
<td>0</td>
<td>2.70</td>
<td>2.43</td>
</tr>
<tr>
<td>Sucrose (%)</td>
<td>4</td>
<td>13.50</td>
<td>12.55</td>
</tr>
<tr>
<td>Corn syrup solids, 36/42 DE (%)</td>
<td>0</td>
<td>6.0</td>
<td>5.4</td>
</tr>
<tr>
<td>Maltodextrins, 10 DE (%)</td>
<td>0</td>
<td>4.0</td>
<td>3.6</td>
</tr>
<tr>
<td>Stabilizer (%)</td>
<td>0</td>
<td>0.70</td>
<td>0.63</td>
</tr>
<tr>
<td>Total solids (%)</td>
<td>15.01</td>
<td>36.97</td>
<td>34.77</td>
</tr>
<tr>
<td>Titratable acidity (%)</td>
<td>0.85</td>
<td>0.15</td>
<td>0.30</td>
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<tr>
<td>pH</td>
<td>4.6</td>
<td>6.7</td>
<td>6.0</td>
</tr>
</tbody>
</table>

is desired in the finished product, the proportion of plain yogurt can be increased to >10% and vice versa.

Soft-serve frozen yogurt may be garnished with nuts and other food materials to enhance its eating experience. The extruded frozen yogurt may be packed in suitable containers and hardened at −25°C to obtain hard-pack frozen yogurt. The ice cream freezer is a scraped surface freezing barrel (heat exchanger). As the liquid mix is pumped through the barrel, removal of the sensible and latent heat leads to formation of frozen mass. The dasher scrapes the inner surface of the barrel while the frozen mass moves toward the exit point. Simultaneously, air cells are formed as a result of whipping action of the dasher and the volume of the mix increases. Eventually, the semifrozen yogurt mass exits from the barrel as foam with a specific controllable degree of aeration. The overrun or the degree of air incorporated in the foam is around 50%. It implies that the original volume of the mix is increased by 50% in the finished frozen yogurt.

The calculation of overrun involves weighing a cup of the mix before freezing and determining the net weight of the mix. Using the same cup, the frozen yogurt is packed and its net weight is determined. The overrun is calculated as follows:

\[
\text{\% overrun} = \frac{(\text{Density of mix} - \text{Density of frozen yogurt}) \times 100}{\text{Density of frozen yogurt}}
\]

\[
= 100 \times \frac{(\text{Net weight of mix} - \text{Net weight of frozen yogurt})}{\text{Net weight of frozen yogurt}}
\]

Assuming the mix weighs 9 pounds per gallon, and the frozen yogurt has 50% overrun, a gallon of frozen yogurt would weigh 6 pounds. Accordingly, one serving of one-half cup (or 4 fl oz) would weigh 85 g.

The shelf life of yogurt may be extended by heating yogurt after culturing to inactivate the culture and the constituent enzymes. Heating to 60–65°C stabilizes the product so the yogurt shelf life will be 8–12 weeks at 12°C. However, this treatment destroys the “live” nature of yogurt, which may be a desirable consumer attribute to retain (Chandan, 2016a).

### 2.5 NUTRIENT PROFILE OF POPULAR COMMERCIAL YOGURT PRODUCTS

Yogurt products provide significant levels of nutrients to the consumer. Table 2.5 enumerates nutrient composition of more popular yogurt products. It is evident that Greek yogurt provides significantly more protein (2–3 times) as compared to regular yogurt. Higher protein in Greek yogurt explains its popularity with consumers.

Various milk constituents of nutritional significance are proteins, fat, lactose, and minerals. Milk proteins have high essential amino acid content. Thus they complement and balance the amino acids composition of relatively lower quality of several vegetable proteins in human diet. The role of major and minor constituents of milk in human nutrition is intertwined with newly discovered physiological benefits. Beyond basic nutrition live and active yogurt possesses additional health benefits, which are discussed in other chapters in this book.
<table>
<thead>
<tr>
<th>Nutrients and Units</th>
<th>Plain, Whole Milk, 8g Protein/8oz</th>
<th>Plain Yogurt, Fruit, Low Fat, 12g Protein/8oz</th>
<th>Plain, Skim Milk, 13g Protein/8oz</th>
<th>Yogurt, Greek, Plain, Whole Milk</th>
<th>Yogurt, Greek, Plain, Low Fat</th>
<th>Yogurt, Greek, Plain, Nonfat</th>
<th>Yogurt, Greek, Fruit, Whole Milk</th>
<th>Yogurt, Greek, Strawberry, Low Fat</th>
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<td>01117</td>
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<td>75.30</td>
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<td>59</td>
<td>106</td>
<td>103</td>
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<td>Protein, g</td>
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<td>5.25</td>
<td>5.73</td>
<td>3.98</td>
<td>9.00</td>
<td>9.95</td>
<td>10.19</td>
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<td>Fat, g</td>
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<td>0.18</td>
<td>1.15</td>
<td>5.00</td>
<td>1.92</td>
<td>0.39</td>
<td>3.00</td>
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<td>Saturated fatty acids, g</td>
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<td>0.116</td>
<td>0.742</td>
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<td>0.117</td>
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<td>Moisture</td>
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<td>0.486</td>
<td>0.053</td>
<td>1.265</td>
<td>0.650</td>
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<tr>
<td>Energy, kcal</td>
<td>0.092</td>
<td>0.044</td>
<td>0.005</td>
<td>0.033</td>
<td>0.469</td>
<td>0.076</td>
<td>0.012</td>
<td>0.219</td>
<td>0.113</td>
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<tr>
<td>Protein, g</td>
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<td>0.044</td>
<td>0.005</td>
<td>0.033</td>
<td>0.469</td>
<td>0.076</td>
<td>0.012</td>
<td>0.219</td>
<td>0.113</td>
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<td>2</td>
<td>5</td>
<td>13</td>
<td>10</td>
<td>5</td>
<td>10</td>
<td>12</td>
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<tr>
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<td>4.66</td>
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<td>7.68</td>
<td>18.64</td>
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<td>3.94</td>
<td>3.60</td>
<td>12.29</td>
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<td>183</td>
<td>199</td>
<td>138</td>
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<td>Calcium, mg</td>
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<td>144</td>
<td>157</td>
<td>109</td>
<td>135</td>
<td>135</td>
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<td>Iron, mg</td>
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<td>36</td>
<td>37</td>
<td>33</td>
<td>33</td>
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<tr>
<td>Magnesium, mg</td>
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<td>46</td>
<td>70</td>
<td>53</td>
<td>35</td>
<td>34</td>
<td>36</td>
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<td>Phosphorus, mg</td>
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<td>0.9</td>
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<tr>
<td>Potassium, mg</td>
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<td>0.9</td>
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<td>0.5</td>
<td>0.4</td>
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<td>Sodium, mg</td>
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<td>0.2</td>
<td>0.8</td>
<td>0.5</td>
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<td>Zinc, mg</td>
<td>0.32</td>
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<td>7</td>
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<td>9</td>
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<tr>
<td>Vitamin C, mg</td>
<td>0.32</td>
<td>0.049</td>
<td>0.053</td>
<td>0.037</td>
<td>0.063</td>
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<td>0.063</td>
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</tr>
<tr>
<td>Thiamin, mg</td>
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<td>0.049</td>
<td>0.053</td>
<td>0.037</td>
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<td>0.055</td>
<td>0.063</td>
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<tr>
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<td>Niacin, mg</td>
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<td>0.063</td>
<td>0.049</td>
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</tr>
<tr>
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<td>0.049</td>
<td>0.053</td>
<td>0.037</td>
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<td>0.055</td>
<td>0.063</td>
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<tr>
<td>Folate, DFE, μg</td>
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<td>0.055</td>
<td>0.063</td>
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<td>0.053</td>
<td>0.037</td>
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<td>0.063</td>
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<tr>
<td>Vitamin A, RAE, μg</td>
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<td>0.049</td>
<td>0.053</td>
<td>0.037</td>
<td>0.063</td>
<td>0.055</td>
<td>0.063</td>
<td>0.049</td>
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<tr>
<td>Vitamin A, IU</td>
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<td>0.053</td>
<td>0.037</td>
<td>0.063</td>
<td>0.055</td>
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<tr>
<td>Vitamin D, IU</td>
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</tr>
<tr>
<td>Vitamin E, mg</td>
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<td>0.037</td>
<td>0.063</td>
<td>0.055</td>
<td>0.063</td>
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<tr>
<td>Vitamin K, μg</td>
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<td>0.049</td>
<td>0.053</td>
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<td>0.063</td>
<td>0.055</td>
<td>0.063</td>
<td>0.049</td>
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</tr>
</tbody>
</table>

Calories factors: Protein 4.27, Fat 8.79, Carbohydrate 3.87.
REFERENCES


3 STABILIZERS, COLORANTS, AND EXOPOLYSACCHARIDES IN YOGURT

Kunal M. Gawai, Sreeja P. Mudgal, Jashbhai B. Prajapati
Anand Agricultural University, Anand, India

3.1 INTRODUCTION

Yogurt is one of the most popular fermented dairy products produced worldwide. It has great consumer acceptability due to its nutritional value and potential health benefits (Weerathilake et al., 2014). It is also considered a very good medium for delivering functional ingredients such as probiotics, prebiotics, and others. Yogurt originated perhaps 10,000–15,000 years ago, probably in the Middle East from adventitious contamination by lactic acid bacteria in milk. Yogurt has been said to have evolved in Turkey as the term yogurt is derived from a Turkish verb, jugurt, that means “curdled or coagulated” (Weerathilake et al., 2014). Tamime and Robinson (1999b), Chandan (2006a), Prajapati and Nair (2008), and Vasiljevic and Shah (2008) have provided exhaustive reviews on accounts of the origins and development of yogurt and other fermented milk products.

In general, yogurt is considered as a nutritionally dense food. It is rich in available protein, calcium, milk fat, potassium, magnesium, vitamin B\textsubscript{2}, B\textsubscript{6}, and B\textsubscript{12} (Ayar and Gürlin, 2014). In addition, it provides milk proteins with a higher biological value and provides almost all the essential amino acids necessary to maintain good health (Lourens-Hattingh and Viljoen, 2001). Yogurt is considered as a probiotic carrier food that can deliver significant amounts of probiotic bacteria in the body and that can provide specific health benefits once ingested (Castro et al., 2015). These are usually marketed as bio- yogurts. Moreover, yogurt is reported to claim improved lactose tolerance, immune enhancement, and prevention of gastrointestinal disorders (Ndife et al., 2014). Because of these known health benefits, consumer demand for yogurt and yogurt-related products has increased and became the fastest growing dairy category in the global market of fermented milk products (Weerathilake et al., 2014). Huge increases in sales of yogurt have been contributed to by variants like Greek yogurt, fresh yogurt, and frozen yogurt (Rule, 2015).

According to the Code of Federal Regulations of the United States, Food and Drug Administration (FDA), yogurt can be defined as a food produced by culturing lactic acid–producing bacteria, \textit{Lactobacillus delbrueckii} ssp. \textit{bulgaricus} and \textit{Streptococcus thermophilus} with one or more of the optional dairy ingredients, namely, cream, high-fat milk, partially skimmed milk, and skim milk (Weerathilake et al., 2014).

Manufacturing yogurt is an ancient technique and knowledge about it has been transferred from generation to generation. Dahi is a similar product from the Indian subcontinent, which appeared before yogurt (Prajapati and Sreeja, 2013). However, during the last few decades, it’s production became more...
regular due to emerged technologies and involvements of various fields such as microbiology, biochemistry, and food and bioengineering. Today, yogurt making is a complex activity combined with art and science. The common types of yogurt are set, stirred, and drinking yogurt, and they are found in most developed markets around the world. The production steps in the manufacture of stirred and set yogurt are illustrated in Fig. 3.1. For all types of yogurt, the first step is the preparation of a yogurt mix and the heat treatment of that mix. The yogurt mix is formulated to have the required amount of milk fat, milk protein, and other nonfat milk solids, and in some countries added sugars, stabilizers, flavors, and colors, if these are permitted. The solids-not-fat content and particularly the protein level affect the texture and firmness of the set and stirred yogurt.

Traditional yogurt should not contain anything other than milk and the starter culture. However, several varieties of yogurt contain additives such as stabilizers, fruits, flavors, colors, sweetening agents, and preservatives. Stabilizers are usually added to the mix in order to increase the body and texture properties, leading to an increase in firmness, reduction in syneresis, and uniform distribution

FIGURE 3.1
Flow chart of yogurt production.
of ingredients. In addition, sweeteners are added to improve the flavor and consumer appeal. Yogurt is made with a variety of other ingredients including fruit pieces, nuts, puree, and colors.

Among the various additives, stabilizers, colorants, and exopolysaccharides have special importance and the fermented dairy products industry is focusing on the use of stabilizers based on their quality and ability to improve texture in combination with other attributes. In the case of colorants, the trend is charging rapidly toward natural colors rather than artificial colorants. Among the natural stabilizers, exopolysaccharides produced by lactic acid bacteria are gaining momentum for use as stabilizers or thickeners. This chapter particularly will focus on polysaccharides (mainly plant originated, enzymatically and chemically derived stabilizers), food colorants, and microbial synthesized exopolysaccharides for use in yogurt production.

### 3.2 STABILIZERS

The primary purpose of adding stabilizers in yogurt is to improve consistency and build viscosity, which helps to minimize whey separation and bind free water. Stabilizers construct the gel matrix and thus increase the stabilization of protein molecules by increasing the water-binding capacity of milk components, mainly casein proteins (Jung, 2002). Stabilizers increase shelf life of the product by reducing water activity and thus inhibiting the growth of undesirable microorganisms. It also minimizes variations among batches, which helps in quality maintenance (Keogh and O’Kennedy, 1998). Low-fat yogurt varieties benefit by the addition of stabilizers. Low-fat yogurts are increasingly popular due to their nutritional and potentially therapeutic characteristics. However, fat reduction may lead to some defects in yogurt such as lack of flavor, weak body, and poor texture (Zhang et al., 2012).

A good yogurt stabilizer should possess characteristics such as not imparting any flavor, effective at low pH values, and easily dispersed at normal temperatures in a yogurt mix (Hoefler, 2004). In addition, stabilizers should be easily soluble, possess good water-holding capacity, and aid in forming stable emulsions. Furthermore, stabilizers improve gelation and adhesion in the case of whipped varieties of yogurt (Kumar and Mishra, 2004; Tavakolipour et al., 2014). Usually, the stabilizer is incorporated using a high shear-type blender that gives strong agitation resulting in complete dispersion and a uniform suspension. Generally, the stabilizers are dispersed in granulated sugar or with nonfat dry matter while being added to minimize potential lump formation. Once dispersed in the mix, it is necessary to have continuous agitation to keep the stabilizer in suspension until it is fully hydrated while receiving proper heat treatment. For choosing the best stabilizer for yogurt making, the following points should be considered (Phillips and Williams, 2009).

1. Variety of yogurt being produced (set, stirred, fruit/pulp added, additives blended)
2. Composition of the yogurt mix (fat and total solids content, strained)
3. Body, texture, and consistency of the finished product desired
4. Labeling on yogurt like natural, organic, kosher
5. Based on batch or continuous type of heat processing of the mix
6. Availability of facility for dosing and mixing, cooling, and pumping of coagulum, i.e., manual or in-line type
7. Intention of masking effect creation on the flavoring system (Chandan and O’Rell, 2006)
Stabilizers are sometimes referred to as hydrocolloids and have two basic functions in yogurt, i.e., to bind the free water and improve the texture (Phillips and Williams, 2009; Hussein et al., 2011). Additives that are normally used as yogurt stabilizers are starches, pectin, gelatin, locust bean gum, guar gum, seaweed gums like alginate and carrageenan, tragacanth, gum arabic, karaya gum, methylcellulose derivatives, xanthan gum, carboxymethylcellulose, and whey protein concentrates (Chandan, 2004; Robinson et al., 2006; Sohail et al., 2014).

### 3.2.1 TYPES OF STABILIZERS

Locust bean gum, carboxyl methylcellulose, guar gum, and alginate are considered as primary stabilizers and are often used in combination with carrageenan (secondary stabilizer) for controlling syneresis. Mucilages and gums of natural origin are also extensively used as binders, thickeners, and suspending and emulsifying agents. These possess more benefits than artificial ones since they are more affordable, nontoxic, as well as readily available (Sohail et al., 2014). Some of the stabilizers that are finding applications in yogurt are presented in Table 3.1 and discussed next.

#### 3.2.1.1 Carrageenan

Carrageenan adds no nutritional value or flavor to foods or beverages. The food industry uses it in several types of yogurt, most notably in squeezable yogurt pouches that are marketed specifically to children. Carrageenan is heat stable and promotes the stabilization of the yogurt gel by complex formation with Ca$^{+2}$ and casein (Chandan, 2004). While the probiotics in yogurt could improve gastrointestinal health, the inflammatory effects of carrageenan could counteract these benefits (Anon, 2014a).

Carrageenan, the “poison ivy of the ocean,” is extracted from red seaweed particularly derived from Irish moss. For the past four decades, scientists have warned that the use of carrageenan in food is not safe.

<table>
<thead>
<tr>
<th>Stabilizer</th>
<th>Sources of Extraction</th>
<th>% Concentration in Yogurt Mix</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whey protein concentrate (34%, 50%, or 80% protein) or and milk protein concentrate</td>
<td>Milk protein concentrate</td>
<td>0.7–1.5</td>
</tr>
<tr>
<td>Starch modified (tapioca/corn)</td>
<td>Seed gums</td>
<td>0.8–2.0</td>
</tr>
<tr>
<td>Gelatin (225/250 bloom)</td>
<td>Protein</td>
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</tr>
<tr>
<td>Agar</td>
<td>Plant extracts</td>
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</tr>
<tr>
<td>Pectin (low methoxy for yogurt)</td>
<td>Pectin</td>
<td>0.08–0.20</td>
</tr>
<tr>
<td>Pectin (high methoxy for yogurt beverages)</td>
<td>Pectin</td>
<td>0.3–0.5</td>
</tr>
<tr>
<td>Locust bean gum (in combination)</td>
<td>Seed gums</td>
<td>0.3–0.5</td>
</tr>
<tr>
<td>Xanthan gum (in combination)</td>
<td>Microbial</td>
<td>0.01–0.05</td>
</tr>
<tr>
<td>Carrageenan (in combination)</td>
<td>Plant extracts</td>
<td>0.01–0.05</td>
</tr>
<tr>
<td>Natural cornstarch</td>
<td>Plant extracts</td>
<td>1.5–2.0</td>
</tr>
<tr>
<td>Carboxymethylcellulose</td>
<td>Cellulosics</td>
<td>0.1–0.2</td>
</tr>
</tbody>
</table>

Table 3.1 Common Stabilizers for Yogurt and Yogurt Drinks With Their Rate of Addition

Animal studies have repeatedly shown that food-grade carrageenan is associated with gastrointestinal inflammation and higher rates of intestinal lesions, ulcerations, and even malignant tumors (Anon, 2014a). However, these reports are not substantiated enough and hence legal authorities have permitted its use in food. It is allowed in the United States by the FDA in organic and nonorganic foods, including juices, chocolate milk, and organic infant formula and considered as safe when used in amounts necessary as emulsifiers, stabilizers, or thickeners. However, the use of carrageenan in infant formula, organic or otherwise, is prohibited in the European Union for precautionary reasons, but is permitted in other food. Furthermore, the Joint FAO/WHO expert committee in 2014 on food additives stated that carrageenan can be used in infant formula for special medical purposes at concentrations up to 1000 mg/L (Anon, 2014b, 2015).

### 3.2.1.2 Pectin

Pectin is made by extracting citrus peel, apple pomace, or beet pulp with hot dilute mineral acid at pH 1.5–3.5. The extract is then filtered, and pectin is precipitated from the clear extract with the synthetic solvents ethanol or isopropanol or as a copper or aluminum salt (Anon, 2007, 2014a). Pectin is used as a popular stabilizer in products that require a natural label perception. However, pectin is expensive and may be used along with locust bean gum as a stabilizer in many fruit-on-bottom-style yogurt preparations (Hoefler, 2004). Pectin is allowed in organic yogurt, even though it is derived from nonorganic fruit. While the organic standards allow the use of pectin, the US Department of Agriculture specifies that only “nonamidated” forms of pectin may be used in organic foods. Amidated pectin is a modified form of pectin. Some of the galacturonic acid is converted with ammonia to carboxylic acid amide. These pectins are more tolerant to varying calcium concentrations, hence they produce firm gel (Belitz et al., 2004). Amidated pectin is produced by suspending dried pectin in alcohol and then treating it with ammonia, which changes the chemical structure of the pectin. Amidated pectin, treated with ammonia, is prohibited in organic yogurt but can be used in conventional yogurt (Anon, 2014a).

Pectins are commonly used alone or in combination with other hydrocolloids to stabilize stirred and set yogurt. The stabilizer system used in yogurt mix preparations is generally a combination of various vegetable stabilizers to which gelatin may be added. Their ratios as well as the final concentration (generally 0.5%–0.7%) in the product are carefully controlled to get the desired effects (Chandan, 2004). Low-methoxy (LM) pectin is the preferred type for (refrigerated) cup yogurt and high-methoxy (HM) pectin is preferred to ensure stability and control viscosity in acidified milk drinks. LM in very small amount (0.07%–0.15%) modifies the consistency of the yogurt making it stiffer and preventing syneresis that might arise during handling, transportation, and distribution. HM pectin stabilizes the milk proteins to produce products without sedimentation, whey separation and ensures a smooth mouthfeel without “sandiness” (Chandan and O’Reill, 2006).

### 3.2.1.3 Gums

Gums are used for stabilization of emulsion, suspension, inhibition of syneresis, and gelling (Sichani et al., 2014). For this reason, they are frequently classified as thickeners and gelling agents. However, this definition is less than perfect because thickeners can sometimes form gels while gelling agents are sometimes used as thickeners (Sanderson, 1996). Classification and function of gums that can be used during the manufacture of yogurt can be found in Fig. 3.2.

Few gums form gels, including kappa- and iota-carrageenan, alginate, agar, and combination between xanthan and locust bean gums. In addition, xanthan produced by *Xanthomonas campestris* was used firstly as a microbial exopolysaccharide (EPS) (Dziezak, 1991; De Vuyst et al., 1998).
Nevertheless, its use is not allowed in most European countries and the United States. Locust bean gum is characterized as a galactomannan and is used for its ability to increase viscosity and prevent fruit flotation and syneresis (Hoefler, 2004).

Among the seed gums, locust bean gum or carob gum is derived from the seeds of a leguminous tree. Carob gum is a neutral polysaccharide and therefore pH has little effect on viscosity in the range pH 3–11. It is insoluble in cold water and must be heated to be dissolved (Chandan and Kilara, 2016). It does not have gelling properties on its own and is used primarily in yogurt to add viscosity or increase gel strength in combination with other stabilizers. It is commonly used in frozen yogurt where its principal function is stabilizing and the binding of water, which provides heat-shock resistance and a slow creamy meltdown (O’Rell and Chandan, 2006). Guar gum is also obtained from seeds and can be used in stabilizer systems for frozen yogurt. Guar gum is readily soluble in cold water and is not affected by high temperatures used in the pasteurization of yogurt mix. Guar gum is nongelling and is used mainly as a viscosity builder, stabilizer, and moisture-binding agent (Yazdanseta et al., 2015). Guar gum imparts body, texture, chewiness, and heat-shock resistance to frozen yogurt (O’Rell and Chandan, 2006). Carboxyl methylcellulose is a derivative of the natural
product cellulose. It is readily soluble in either hot or cold water and is effective at high processing temperatures. Its primary function is as a thickener and moisture-binding agent (Saha and Bhattacharya, 2010). In frozen yogurt, it functions to bind water, thus preventing the formation of large ice crystals that can develop due to temperature fluctuations during storage (Maier et al., 1993). The result is frozen yogurt with smoother texture and improved meltdown characteristics. The stabilizer system used in yogurt mix preparations is generally a combination of various vegetable stabilizers. Their ratios as well as the final concentration (generally 0.5–2.0%) in the product are carefully controlled to get desirable effects (Chandan and O’Rell, 2006).

3.2.1.4 Native and Modified Corn Starch
Modified corn starch is found in many highly processed conventional yogurt products (Anon, 2014a). The most common stabilizer used in processed fruit products as well as in blended yogurts is modified food starch (MFS), usually derived from corn. The only disadvantage is that it may mask flavor release (Chandan, 2006b). It is easy to handle during processing and is cost effective (Tavakolipour et al., 2014). Starch is the most commonly used hydrocolloid thickener and is used both in its native and modified forms (Babic et al., 2009). Cross-linking of starch chains with phosphate diester reduces the degree and rate of granule swelling and helps to stabilize the yogurt and provide resistance to breakdown during mechanical shearing. Modified corn/tapioca starch suitable for use at low pH is commonly used in yogurt formulation. For example, a stabilized and medium cross-linked waxy maize starch (hydroxypropyl distarch phosphate) is a viscosity generator and a stabilizer. It has a bland flavor, gives clear paste, smooth short texture, and can withstand severe processing conditions of low pH, high heat, and extreme shear (Chandan and O’Rell, 2006).

3.2.1.5 Gelatin
Gelatin is added to some yogurt for consistency and texture. Gelatin has a unique meltdown mouthfeel, which makes it different from vegetarian thickeners like pectin. It is a protein found in animal parts such as fish skin or cow and pig hides, tendons, and bones. It is derived by irreversible hydrolysis of the proteins collagen and ossein (Chandan and O’Rell, 2006). In this way, it is a convenient way to utilize the vast quantity of slaughter by-products in food industry. Gelatin in yogurt is often labeled “kosher gelatin,” which refers to Jewish food rules, and makes the yogurt acceptable to Jews who follow kosher laws. Kosher gelatin is derived from an animal or an animal part that was deemed kosher by a rabbi (priest). The basis of kosher gelatin can be fish or the hides of kosher cows. Gelatin has a “generally recognized as safe” (GRAS) status since 1975 but the FDA reconsidered changing this status in 1997 after incidences that showed gelatin can harbor prions of bovine spongiform encephalopathy disease (Anon, 2014a).

Gelatin exhibits a wide range of functional properties. Gelatin gels melt at relatively low temperature (melt in mouth), and they are slow-setting; all these features make gelatin the preferred gelling agent in yogurt products, low-fat spreads, and sugar confectionery (Saha and Bhattacharya, 2010). Gelatin is a well-known ingredient in low-fat yogurts, due to its melting behavior at body temperature. Gelatin has a natural taste, minimum nutritional value (Anon, 2014a), and does not have an E additive number. Tavakolipour et al. (2014) reported that addition of gelatin to the milk during the preparation of yogurt changed the microstructure of the product by the formation of flat sheets or surfaces that interacted with the casein matrix, enclosing granules of casein in several zones. The gelatin seemed to connect the granules and chains of milk proteins, and consequently create a continuous, fairly homogeneous double network structure with no free ends.
Gelatin is used at a level of 0.1–0.5%, depending on the firmness, and at a level of 0.3–0.5% to get a smooth shiny appearance in refrigerated yogurt (Chandan, 2004). Gelatin is a good stabilizer for frozen yogurt as well. Gelatin of bloom strength (unit to measure gel strength) of 225 or 250 is commonly used (Chandan, 2004). If gelatin is added above 0.35% (w/w) it may give yogurt a lump-like appearance upon stirring (Chandan, 2006b).

### 3.2.1.6 Use of Hydrocolloids

Hydrocolloids are hydrophilic molecules that have a high molecular weight. They are used as functional ingredients in food formulation for increasing food consistency, improving gelling effect, and controlling the microstructure, texture, flavor, and shelf life. Hydrocolloids, or more commonly gums, are defined as complex nondigestible polysaccharides that dissolve or disperse in water to give thickening or viscosity building effect (Anderson and Andon, 1988; Bergenstahl et al., 1988). The use of hydrocolloids has been greatly increased in recent years. Hydrocolloids are used at a concentration of less than 1%, which affects the textural properties and mouthfeel characteristics of products. The texture generated from these hydrocolloid compounds like gum seeds is weak. Many studies have been carried out to improve physicochemical and rheological properties of low-fat and nonfat yogurt, with a wide variety of additives in combinations with hydrocolloids (Glickman, 1986).

Though all hydrocolloids thicken aqueous dispersions, only a comparatively few gums form gels. The important gums that find application in food as gelling agents include alginate, pectin, carrageenan, gellan, gelatin, agar, modified starch, methylcellulose, and hydroxypropyl methylcellulose (Saha and Bhattacharya, 2010). Among these additives, multiple functional properties of hydrocolloids make it possible to use them extensively. These features include varying degrees of adhesiveness to flow ability, i.e., viscosity and increasing consistency (Sichani et al., 2014). Hydrocolloids were used in production of soymilk and yogurt (Villaudy et al., 1987; Afaneh, 2013).

### 3.3 COLORS

Color is one of the most important attributes of foods and beverages and it is the first thing someone could notice in a food product. Appeal created by color in foods persuades us to taste the product (Nachay, 2009). Color is added to fruit and flavored yogurts to make the products more attractive (Ulberth et al., 1993). Processing degrades color and color of the product diminishes and loses vibrancy over time because direct or indirect interaction with light, air, moisture, temperature, and storage conditions. Manufacturers usually add a spectrum of color ingredients to create consistency across a product line considering factors affecting coloring integrity aspects within products and legal permitted limits allowed by the authorities. Some of the colors are being added to food products for protecting vitamins like carotene and betalains and flavors like flavonoids against degradation during storage (Nachay, 2009).

### 3.3.1 CLASSIFICATION OF FOOD COLORANTS

The active agents being used in food products may be naturally derived, nature identical or artificially synthesized (Tamime and Robinson, 2007). They may also be classified as (1) water soluble and (2) oil soluble.
3.3.1.1 Natural Colorant

Natural colors are added to enhance appeal with flavors. Common natural colors include annatto, saffron, paprika, grape skins, zinc oxide, caramel, beetroot, cochineal, turmeric, carotenes, chlorophyll, canthaxanthin, riboflavin, curcumin, and many more (Bride and Timberlake, 1997; FDA, 2010). The FDA uses the term “exempt color additives” to indicate those that are exempted from the certification process (FDA, 2010). Exempt colors need to be used at higher levels to compensate for the same intensity effects produced by artificial colors. As a consequence, they may change the texture, odor, or flavor of the food. They are less stable and consistent and may cause preferential consumer acceptability change.

An interesting ancillary use for colors derived from natural sources is that many are bioactive. The antioxidant effects of many green, red, yellow, blue, and orange fruits and vegetables derive their indigenous and quite vivid bioactive colors, such as taxanthin, lycopene, lutein, anthocyanin, and beta-carotene. Nowadays, fermentative production of food grade pigments is available in the market, for example, color from Monascus spp., astaxanthin from Xanthophyllomyces dendrorhous, Arpink red color from Penicillium oxalicum, riboflavin from Ashbya gossypii, and carotene from Blakeslea trispora. Also a number of microorganisms produce biocolors in good amounts, including Serratia and Streptomyces (Kim et al., 1997). As per the Food Safety Authority of India, natural coloring matters that can be used in food products as per good manufacturing practices are carotene and carotenoids, chlorophyll, riboflavin (lactoflavin), caramel, annatto, saffron, curcumin, and turmeric (FSSA, 2006).

3.3.1.2 Synthetic Colors

Synthetic or artificial colors are certified by a legal body and they are expected to have minimum batch-to-batch variation and extensively analyzed by sophisticated techniques to ensure that they meet established specifications for residual contaminants, heavy metals, pesticides, and unreacted contaminants (Griffiths, 2005).

More than 17 million pounds of synthetic food colors were certified in 2015 (Mergel, 2016). FD & C Red No. 40 is the most popular certified food color, followed by FD & C Yellow No. 5. The acronym FD & C indicates that these colors are approved by the FDA for use in coloring foods, drugs, and cosmetics. Certified colors in all three categories may be used as dyes or converted to lakes. Dyes will dissolve in water and can be manufactured as a powder, granule, or liquid. They are often used in aqueous beverages, dry mixes, confections, and dairy products. Lakes, on the other hand, are prepared by precipitating the soluble synthetic dye onto an approved insoluble base or substratum (Griffiths, 2005).

Synthetic colors are of importance as they are widely used in different foods. They are again classified as acidic and basic dyes. Eight coal-tar food colors were permitted to be used in several food products including yogurt under the provisions of the Prevention of Food Adulteration Act in India. However, at present all the permitted water-soluble dyes are acidic in nature and hence indication of basic dye is not logical. However, certain colors such as Metanil yellow, Rhodamine B, Orange G, Blue VRS, Auramine, and certain unidentified water- and oil-soluble colors are not permitted and often appear as adulterants in foods (www.fssai.gov.in). The Food Safety and Standard Authority of India (FSSAI) has restricted use of synthetic food colors or a mixture thereof and the following shall be used in food (Anon, 2005; FSSA, 2006). A list of certified use of colors allowed for use in food products is given in Table 3.2.
3.3.1.3 Color Addition in Yogurt

Flavors and certified colors are usually added to yogurt preparations for improved eye appeal and better flavor profile. The pH control of the base is important for fruit color retention in yogurt, if used. The color of the yogurt should represent the fruit color in intensity, hue, and shade (Chandan, 2004). The color of the fruit preparation is usually used to color the finished yogurt. The options include: (1) no color added, therefore relying on the natural color of the fruit, and (2) color added, natural colors, or artificial colors. Artificial colors (red #40, blue #1, and yellow #5 and #6) are very stable during processing. These are the most economical choice, but have fallen out of favor for labeling reasons.

Natural colors are preferred because of their heat stability during processing, including black carrot, grape extract (Kosher or non-Kosher), chokeberries, elderberry, red cabbage, radish, black current, carmine, annatto, and turmeric. Some of these colors simply appear as “vegetable color” in the ingredient declaration. Beet juice is sometimes used in fruit preparations, but because of its instability during heat processing, many times it will be added during coagulum breaking in case of stirred yogurt or after heat processing in case of set type (O’Reill and Chandan, 2006).

The use of colors in fruit preparations for use in dairy products is universal. Fruit preparations typically make use of yellow, orange, or red colors. Maximum level permitted of food coloring matter arising exclusively from flavoring substances is provided in Table 3.3. However, consumer preferences and industry standards are driving a conversion from synthetic to natural colors in the dairy industry. The natural colors currently used for yellow-orange shades are turmeric, beta-carotene, and paprika, while anthocyanins and carmine typically utilized for red colors. However, some of these colors are unstable when processed at high temperature or at different pH values.

### 3.4 Common Colors Used in Yogurt Preparation

#### 3.4.1 Carmine

Carmine is extracted from the shells of the cochinelle beetle, which is native to semiarid regions of Central and South America. The beetle’s shell contains carminic acid. Carmine pigments are formed
when this carminic acid is precipitated with aluminum calcium salts. When intricate with aluminum, at pH 4, a series of stable brilliant red hues ranging from “strawberry” to “blackcurrant” can be produced depending on the concentration of aluminum (Francis, 2002). Synthesis of Al$^{+3}$ ions mainly due to exposure to aluminum during this process may raise chances to precipitate a variety of neurodegenerative disorders, including Alzheimer’s disease while ingesting this color in the diet. Carmine usually contains about 50% carminic acid. It is of special use to the food industry as it resistant to heat and light and has longer stability during extended storage. It is ideally suited to foods with a pH above 3.5, and it is also used in a wide variety of other products such as jams, gelatin desserts, baked goods, confections, toppings, dairy products, noncarbonated drinks, and many others (Francis, 2002). Yogurt manufacturers like Danone have sometime favored it over artificial colors because it allows the products to appear to be more “natural” (Anon, 2014a).

### 3.4.2 ANNATTO

Annatto is the natural extract from the red oily outer layer of the seeds of a tropical shrub *Bixa orellana*, which is commercially grown for its dye products and for its seeds (Francis, 2002). Individuals who are sensitive to annatto may experience hives or gastrointestinal distress from ingesting it (Anon, 2014a). It is mainly used for butter color in dairy industry but has found a prominent place in yogurt and cheese too.

### 3.4.3 COLORS FROM CONVENTIONAL FRUIT AND VEGETABLES

The most popular flavorings for yogurts are of fruit types that enhance the sensory as well as visual attractiveness of yogurt. Among nontraditional additives are also vegetable powders, pulps, and natural extracts obtained from raw vegetables that have been used in the production of fermented milk.
products (Tamime and Robinson, 1999a). Supplementation of yogurt with selected vegetables will provide additional nutritional health benefits apart from increasing appeal of the product (Najgebauer-Lejko et al., 2014). Nowadays, organically produced fruits and vegetables are being added to yogurt (Anon, 2014a).

Byamukama et al. (2014) used anthocyanins from mulberry (Morus rubra) fruits as potential natural color additives in yogurt at the rate of 25–40 mg per 100 mL and found the quality of the product to be acceptable. Addition of strawberries and their extracts are a popular practice to impart colors as well as nutrition to yogurt (Thompson et al., 2007; Allgeyer et al., 2010). Carrot juice is also used by yogurt industry to impart color and nutritional richness to yogurt. Excellent quality carrot-yogurt could be prepared by blending milk in different proportions having 5%–20% carrot juice before fermentation (Sharma et al., 2012). Many researchers have mentioned fortifications with bioactive components like caffeine, guarana, green tea extract, coenzyme Q10, ginseng, aloe vera, fruit pulp, fiber, and oat in yogurt preparations (Gawai and Prajapati, 2012; Najgebauer-Lejko et al., 2014; Prajapati et al., 2014).

### 3.5 EXOPOLYSACCHARIDES

The use of cultures producing EPS plays an important industrial role in the textural development of yogurts and other fermented milk products, low-fat cheeses, and in dairy desserts (Gürsoy et al., 2010). The EPSs produced by yogurt starter cultures affect the textural and physical properties of low-fat yogurt and improve the sensory characteristics such as mouthfeel, shininess, clean-cut, ropiness, and creaminess (Ruas-Madiedo et al., 2005; Folkenberg et al., 2006). Both capsular and ropy EPS possess high water-binding ability, which results in increased water retention in yogurt (Hassan et al., 1996). Additionally, EPS has been reported to provide physiological benefits such as lowering of cholesterol, immunomodulation, and antitumor activity (Welman and Maddox, 2003; Lin and Chang Chien, 2007; Purwandari and Vasiljevic, 2010). Therefore, use of EPS-producing cultures particularly in developing low-fat yogurt with physiological functions is important (Purwandari and Vasiljevic, 2010; Zhang et al., 2012). EPSs from lactic acid bacteria have certain health benefits for consumers. High viscosity increases the retention time that the fermented product spends in the gastrointestinal tract, which helps the colonization of starter bacteria used for yogurt making and probiotic bacteria. EPSs can be metabolized by the colonic microorganisms to form short-chain fatty acids like acetate, propionate, and butyrate, and those cannot only provide energy to epithelial cells but also play a role in the prevention of colon cancer (Lin and Chang Chien, 2007). Because of their role in health enhancement, some microbial polysaccharides are sometimes defined as prebiotics, which are defined as “non-digestible 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” (Stefanović et al., 2012).

Polysaccharides generally being used in fermented foods are viscosifying agents, stabilizers, emulsifiers, gelling agents, and water-binding agents (Looijesteijn et al., 2000). However, most of them are chemically or enzymatically modified in order to improve their rheological properties, therefore, their use is strongly restricted for food applications.

An alternative source of biopolymers that can improve viscosity in food matrix is microbial EPS. The EPSs of microbial origin have unique rheological properties because of their capability to form very viscous solutions at low concentrations and their pseudoplastic nature. Inherently, lactic acid
bacteria (LAB), which are GRAS, are associated with many fermented foods, particularly milk-based products such as curd, yogurt, sour cream, cheese, and buttermilk, where they contribute basically to develop taste, flavor, and shelf life, which allows them to be incorporated in food without labeling. Some strains of LAB have been reported to produce EPS and have drawn attention over the last few years because of their potential contribution in improving texture of fermented milk (Mende et al., 2011). Most of the EPSs producing LAB belong to the genera *Streptococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, and *Pediococcus* and from some strains of nonstarter LAB or adjunct bacteria like *Bifidobacterium*. LAB are able to produce mainly two types of polysaccharides according to their location in the cell, intracellular polysaccharides, and extracellular polysaccharides. Some bacteria produce only capsular EPS, some produce only slime (ropy) form (Zisu and Shah, 2005, 2007; Folkenberg et al., 2006), whereas, in some cases, bacteria can produce both forms of EPS (Cerning, 1995; Patel and Prajapati, 2013; Feldmane et al., 2014; Bunkoed and Thaniyavarn, 2014). EPSs fill the pores in the protein network in yogurt, with more-ropy EPS showing a more compact strand-like structure than less-ropy EPS (Hassan et al., 2003).

A high consumer demand for products containing low-sugar content, low levels of food additives, especially stabilizers and thickeners, makes EPS a feasible alternative (Welman and Maddox, 2003; Behare et al., 2009; Mende et al., 2011; Feldmane et al., 2013).

The amount of EPS produced and its properties are strain dependent. EPS production by different yogurt starters varies roughly from 45 to 350 mg/L. Exopolysaccharides resulting from *S. thermophilus* and *L. delbrueckii ssp. bulgaricus* show large variations in composition, charge, spatial arrangement, rigidity, and ability to interact with proteins (Feldmane et al., 2013).

### 3.5.1 Natural EPS Production in Yogurt

A combination of *S. thermophilus* and *L. delbrueckii ssp. bulgaricus* that grow synergistically is widely being used as starter cultures for the production of yogurt. They have importance not only in view of their ability to acidify, which preserves yogurt from spoilage, but also to make a major contribution to its organoleptic property (Tamime and Robinson, 1985). It is widely known that these starters secrete exopolysaccharide.

Growth of starter culture in fermented products requires sufficient proteolytic activity. In yogurt, *S. thermophilus* is described as a poor proteolytic species, which requires several essential amino acids for its growth mainly, valine apparently being the most important (Tamime and Robinson, 1985). *L. delbrueckii ssp. bulgaricus* provides the amino acids through its proteolytic activity. The synergistic interaction between the two cultures is also expressed in their EPS production, which gradually increases viscosity during yogurt production (Moreira et al., 2000).

In *S. thermophilus*, apparently the energy-generating system in the presence of galactose operates at a slower rate resulting in a slower cell growth, compared to that of other sugars. In this species, the main product of Leloir pathway is sugar nucleotide, a monomer for EPS polymer building (Boels et al., 2001). In *L. delbrueckii ssp. bulgaricus*, however, the presence of galactose-metabolizing enzymes and Leloir pathway may not always relate to EPS production (Marshall et al., 2001). As a consequence, the EPS production is less stable and easily altered by external factors such as sugar type, temperature, and other supplements (Boels et al., 2001). In general, strains of *S. thermophilus* possess the Leloir pathway; however, the system varies in the activity rate. In Gal– strains, the enzymes in the system are less active than in Gal+ strains (Degeest and De Vuyst, 2000).
3.5.2 CLASSIFICATION OF EPS
EPS are classified into two groups: homo-EPS, consisting of a single type of monosaccharide (α-d-glucans, β-d-glucans, fructans, and others represented by polygalactan) and hetero-EPS, consisting of regular repeating units of 3–8 different carbohydrate moieties, mainly d-glucose, d-galactose, L-rhamnose, and their derivatives synthesized from intracellular sugar nucleotide precursors (Cerning, 1990; De Vuyst and Degeest, 1999; Bunkoed and Thanilyavarn, 2014; Patil et al., 2015).

During the early part of the EPS studies, it was found that EPS was a molecule like protein. EPS-producing lactic acid bacteria can produce not only one type of polysaccharide but also different types of polysaccharides due to fermentation conditions. In addition, it is possible that the same strain is able to produce high-molecular-mass and low-molecular-mass EPS fractions that do not differ in monomeric composition (De Vuyst and Degeest, 1999). The biosynthesis of homo-EPS and hetero-EPS are different. Homo-EPS is made from sucrose using glucansucrase or levansucrase, and the synthesis of hetero-EPS involves four major steps: sugar transportation, sugar nucleotide synthesis, repeating unit synthesis, and polymerization of the repeating units (Harutoshi, 2013).

3.5.2.1 Homo-EPS
Homopolysaccharides are the polymers composed of one type of monosaccharide (Vaningelgem et al., 2004b). Homopolysaccharides are present in the form of glucans, which mainly contain α-1,6 and α-1,3-linked glucose residues, namely dextrans, produced by Leuconostoc mesenteroides ssp. mesenteroides and Leuconostoc mesenteroides ssp. dextranicum; fructans, composed of β-2,6-linked d-fructose molecules like levan produced by S. salivarius, L. mesenteroide, and Streptococci, and finally others that are mainly polygalactans composed of structurally identical repeating units with different glycosidic linkages and mutants produced by Streptococcus mutans and Streptococcus sobrinus. Homo-EPS is synthesized in the presence of a specific substrate such as sucrose (De Vuyst and Degeest, 1999).

3.5.2.2 Hetero-EPS
Heteropolysaccharides are the polymers of repeating units that are composed of two or more types of monosaccharides (Degeest et al., 2001; Welman and Maddox, 2003; Vaningelgem et al., 2004b). Heteropolysaccharides synthesis is different from homopolysaccharides synthesis due to formation of intracellular precursor repeating units and having isoprenoid glycosyl carried lipids in the process (Ruas-Madiedo and de los Reyes-Gavilan, 2005) and mainly governed by monosaccharide composition, linkage types between polymer units, branching, molecular weight, monosaccharide charge, and also by the ability to interact with milk proteins (Vaningelgem et al., 2004a). The structure of the repeating unit of a LAB heteropolysaccharide produced by S. thermophilus was first determined by Vaningelgem et al. (2004a).

3.5.3 EPS IN YOGURT
Feldmane et al. (2014) measured EPS production during yogurt fermentation. The production of intracellularly synthesized EPS varies roughly from 25.28 to 440.81 mg/L during fermentation. The fermentation temperature significantly contributes to EPS concentration because the increased rate of fermentation temperature is attributed to increased metabolic activity of LAB. The quantities of EPS produced in milk by different species and strains vary considerably; the amount of EPS reportedly
ranges from 50 to 350 mg/L for *S. thermophilus*, from 60 to 150 mg/L for *L. delbrueckii* ssp. *bulgaricus*, and from 80 to 600 mg/L for *L. lactis* ssp. *cremoris* (Cerning, 1995).

Mozzi et al. (1995) demonstrated an increased EPS production from *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* at incubation temperatures of 32°C or 37°C instead of 42°C. EPS production from mesophilic lactic acid bacteria is almost 50% higher when the organisms are grown at 25°C instead of 30°C (Cerning et al., 1992). For some strains of *L. lactis* ssp. *cremoris*, temperatures as low as 18°C have been chosen to enhance EPS synthesis (Kontusaari and Forsen, 1988). Adjustment of the pH to 6.0 of the whey-based fermentation medium for growth of *S. thermophilus*, *L. delbrueckii* ssp. *bulgaricus*, and *L. lactis* supposedly promotes EPS formation as relative viscosities increase (Cerning, 1995). Many of the investigations on EPS formation by starter cultures used in the manufacture of yogurt and fermented milk (*S. thermophilus*, *L. delbrueckii* ssp. *bulgaricus*, *L. lactis* ssp. *cremoris*) have been carried out with milk (i.e., lactose being the fermentable sugar). Neither growth nor EPS production was specifically linked to the presence of casein or whey proteins in the medium for thermophilic and mesophilic lactic acid bacteria. Casein stimulates EPS production but not growth of *L. delbrueckii* ssp. *bulgaricus* (Cerning, 1995). It has also been reported that *L. delbrueckii* ssp. *bulgaricus* is able to produce the same amount of EPS in milk and milk ultrafiltrate, but that *S. thermophilus* cannot (Cerning et al., 1990).

The EPS-producing cultures are commonly used as an alternative to minimize or eliminate use of commercial stabilizers in yogurt manufacturing. Some researchers reported that some EPS-producing (ropy) lactic acid bacteria showed a higher viscosity and less syneresis, which is in contrast to non-EPS-producing lactic acid bacteria (Bouzar et al., 1996; Folkenberg et al., 2006). EPS-producing lactic acid bacteria have gained popularity as being safe and are used as natural stabilizers. Moreover, recently there is increasing interest for using EPS-producing dairy cultures because of high consumer demand for smooth and creamy yogurt products (Welman and Maddox, 2003).

The EPS content in yogurt affects the yogurt texture. In general, it reduces firmness of yogurt (Folkenberg et al., 2006). Ropy type of EPS gives rise to ropiness of yogurt, as well as mouth thickness and creaminess. A mechanism for fat replacer capacity of EPS has been suggested. This involves the ability of EPS to cover the tongue and longer feeling of EPS by delaying the cleaning of mouth by saliva (Cayot et al., 2008). The thickness character is raised from a high cohesion among yogurt particles, resulting in a more elastic material. Mouth thickness is correlated with viscosity at shear rate of 241/s (Folkenberg et al., 2006). Ropiness of EPS is related to creaminess, although the EPS produced by some nonropy strains are also able to exhibit creaminess in yogurt (Folkenberg et al., 2006). The textural properties depend on EPS produced by different strains. The EPS from *L. delbrueckii* ssp. *bulgaricus* influenced the firmness while that produced by *S. thermophilus* gave creaminess, mouth thickness, and ropiness of the final product (Folkenberg et al., 2006).

Among the traditional fermented milk products, yogurt and its varieties are considered a major contributor to global sales revenue. Yogurt industry is flourishing steadily and expecting huge turnout in coming decades. At the same time, consumers are expecting more and more of variety and value addition to existing products and more of natural and organic options. Over the past decade the use of natural additives in food and beverages has increased to a greater extent than that.
of synthetic alternatives. Industries are trying for combinations of stabilizers to get added benefits such as flavor, color, and nutritional enhancement or maybe added health benefits simultaneously. At the same time the regulatory aspects of incorporation of these additives should be carefully considered. Natural additives such as use of bacterial cultures for production of EPS are another option. Such EPS strains could be useful as a thickener as well as fat replacer. However, the search for bacteria with high exopolysaccharide yields is an ongoing process. The manipulation of fermentation conditions, genetic and metabolic engineering, as well as the exploration of fermentation process further are suggested tools for improving the characteristics of yogurt and similar products.

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REFERENCES


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Chapter 4

MICROBIOLOGY OF YOGURT AND BIO-YOGURTS CONTAINING PROBIOTICS AND PREBIOTICS

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4.1 INTRODUCTION

It is believed that humans have been consuming milk products for almost 7000–12,000 years (Moreno Aznar et al., 2013). Fermented milk was first developed in the Middle East when it was stored in animal stomachs; the enzymes naturally present curdled the milk making what was essentially yogurt. This practice was thus used for preservation purposes but the different flavors and textures led to what would eventually become today’s yogurt industry.

The actual yeasts and bacteria responsible for yogurt production were identified by the French chemist and microbiologist Louis Pasteur in the 19th century. Other groups around the world helped to classify these organisms as lactic acid bacteria. The key functional elements of starter cultures of lactic acid bacteria include: preservation of the product; development of flavor, texture, and aroma characteristics (organoleptic); and to enhance nutritional quality. In recent years, there has been increased interest in producing fermented foods whose starter cultures are capable of more than these traditional values by improving the health of the consumer. However, the concept of yogurt as a health food has been with us since the beginning of the 20th century when Metchnikoff attributed the relatively long life span of Bulgarian peasants to their regular consumption of yogurt, which was not due to the yogurt itself but rather to the bacteria it contained. His research led him to believe that \textit{Lactobacillus} was essential for good health and encouraged yogurt consumption on a regular basis throughout Europe. The industrialization of yogurt production first took place in Barcelona in 1919 where Isaac Carasso set up a small yogurt business named Danone (later to be branded Dannon when it expanded into the United States).

In recent years, major research has been invested in increasing or maximizing the potential health benefits of yogurt and fermented milk drinks. This is largely driven by our knowledge of the role of the microbiota in human health, which is an attractive target for novel therapeutics through the use of functional foods (Ejtahed et al., 2016). Consequently, there is heightened interest in probiotics and prebiotics as additions to milk not to impact the starter cultures or yogurt but rather to benefit the consumer.

Although yogurt is a common food for large parts of the world, its perception as a basic product makes it an underappreciated food to consumers in terms of health attributes. Yogurt holds great
potential as a delivery vehicle for a wide range of health benefits exacted through modulation of the intestinal microbiota (Martinez et al., 2015). This chapter will discuss the current views and future directions for probiotics and prebiotics in yogurt products and address some of the current regulatory issues.

### 4.2 Manufacture and Microbiology of Yogurt

Yogurt is made through fermentation of milk via the action of starter bacteria, namely *Lactobacillus delbrueckii* ssp. *bulgaricus* (*Lactobacillus bulgaricus*) and *Streptococcus thermophilus*, with or without the addition of other optional ingredients. The World Health Organization (WHO)/Food and Agriculture Organization (FAO) definition states that the microorganisms in the final product must be viable and abundant (WHO/FAO, 2011).

Yogurt can be characterized as set or stirred; set yogurt is formed when the coagulation of the milk is completed in the individual retail container, while stirred yogurt is produced when the coagulum is broken in the incubation tank and slowly cooled and packaged. Yogurt-derived products include drinking yogurt, frozen yogurt, dried yogurt, low-fat yogurt, and bio-yogurt. Bio-yogurts, for example, Activia, contain probiotics such as *Bifidobacterium*, which is not part of the starter.

The milk used in yogurt production should be of excellent microbiological quality, be free of any inhibitory substances, as *S. thermophilus* is very sensitive to penicillin, and contain at least 8.5% solids-not-fat (SNF). The consistency and aroma of the yogurt is improved by increasing the total solids: by adding milk powder or evaporating off water. In some cases stabilizers such as gelatin may also be added to improve viscosity, though this is now possible through the use of prebiotics (Hernandez-Hernandez et al., 2011).

The yogurt milk is usually inoculated with the starter cultures at a rate of 2%–3% and incubated at 40–45°C. At this temperature range, the final desired acidity is attained in 3–4 h. A lower temperature of 30–37°C over a longer time (7–8 h) may be used in some cases to promote slime production and prevent overacidification. This slime is usually due to exopolysaccharide (EPS) production from the *S. thermophilus* culture and provides extra viscosity (Hassan et al., 2003).

The final coagulum should have a shiny appearance, a smooth texture and a custard-like consistency. These characteristics describe the rheology of the yogurt product. The acidity of the yogurt will affect viscosity, while the rate of acid production also affects the physical characteristics of the gel; in this regard an even rate of acid production is desired.

The cooling of the yogurt is a critical step as it restricts further starter growth, slows/stops enzymatic activity and prevents overacidification. The temperature should be lowered to below 10°C. Stirred yogurt may be agitated at various temperatures before the final cooling temperature is achieved. This must be done carefully to ensure the yogurt is handled very gently to avoid defects in yogurt consistency and viscosity.

### 4.3 Starter Cultures

*S. thermophilus* is mostly responsible for the flavor, aroma and texture of the yogurt, and is, in fact, capable of making yogurt on its own. The role of *L. bulgaricus* is to produce acid. *S. thermophilus* and *L. bulgaricus* are usually inoculated simultaneously into the yogurt milk (pH 6.6) in a 1:1 ratio, which changes as the fermentation progresses. Cocci predominate when acidity is low while the *Lactobacillus*
increases when acidity levels are higher due to their greater acid tolerance. In addition, lower temperatures and inoculum levels favor growth of cocci. Most yogurts are now made using concentrated frozen or freeze-dried defined cultures (Huang et al., 2006).

The composition of the yogurt milk also impacts on the growth of the starter whereby SNF, fat content, and concentration of mineral salts can influence the rate of acid production and flavor and aroma development. High SNF gives a high buffering capacity, which results in starter cells remaining viable for a longer time. Sucrose (typically 5%–6% is added) in excess of 9% inhibits culture growth, and acid and acetaldehyde production, although *S. thermophilus* is more tolerant of high-sugar concentrations.

The severe heat treatment of the milk is also a factor affecting culture growth, breaking protein into peptides and amino acids, which encourage *S. thermophilus* growth. Interestingly, *S. thermophilus* and *L. bulgaricus* proliferate much faster in milk when cultured together than when either is grown separately. In this regard, they have a synergistic relationship termed protocooperative growth. *S. thermophilus* produces the enzyme urease, which releases formic acid and CO$_2$ from urea in milk. Formic acid stimulates *L. bulgaricus*, and CO$_2$ also encourages growth by creating a more anaerobic environment. On the other hand, *L. bulgaricus* is the more proteolytic of the two starter cultures and generates peptides and amino acids that stimulate *S. thermophilus* growth.

## 4.4 PROBIOTICS

Probiotic bacteria are generally agreed to have three basic mechanisms by which they can impact human health (Segers and Lebeer, 2014). Firstly, they can exclude pathogens through influencing the commensal microbiota, or by direct action against the pathogen; secondly by enhancing the barrier function of the epithelial layer; and thirdly through modulation of the host immune responses, causing local and systemic effects. One particular probiotic trait, bacteriocin production, offers an advantage to both the food and the consumer. Bacteriocins are ribosomally synthesized antimicrobial peptides that are produced by a wide range of bacterial species and constitute the antimicrobial mechanism of some probiotics. Bacteriocins can have a narrow or broad spectrum of activity, for example, nisin is effective against a broad range of gram-positive pathogens (Le Blay et al., 2007). These peptides can be added directly to the food product as in the case of nisin or can be produced in situ by the probiotic culture, where either way they provide protection against food spoilage and pathogenic microorganisms (Cotter et al., 2005). In the gut bacteriocins are thought to have three potential mechanisms by which they promote probiotic functionality: (1) act as colonizing peptides, conferring a competitive advantage to the probiotic, (2) act as killing peptides, directly acting upon the pathogenic microorganism, (3) act as signaling peptides to the immune system or other bacteria (O’Shea et al., 2012).

The field of research in probiotics is relatively recent and so the definitions and conditions for evaluation are constantly evolving with new data coming to light. There is currently no statutory or regulatory definition for the term *probiotic*. In 2001, the joint FAO and WHO Expert Consultation on “Evaluation of Health and Nutritional Properties of Probiotics in Food including Powder Milk with Live Lactic Acid Bacteria” proposed a definition that is the most widely accepted: “live microorganisms that, when administered in adequate amounts, confer a health benefit on the host.” While this is widely accepted and used, it is of the utmost importance in the near future that a legal definition is established to ensure the scientific community and food industries are working under the same one to avoid misleading information on food products. However, it is important to note that this definition is potentially out of date due to literature that shows that one possible probiotic
mechanism of action involves the stimulation of the immune system and in this instance it is debatable whether the probiotic organism needs to be “alive” to induce such an effect (Sanders, 2008). Without a legal definition, the mislabeling of products (which do not meet the criteria accepted by the scientific community) is occurring, and as public awareness grows it is increasingly important to ensure any product labeled as a probiotic meets the correct criteria (Goldstein et al., 2014). Without correct regulation consumers are likely to lack confidence in any probiotic claim in the future (Vandenplas et al., 2015). While a legal definition is important to prevent the category from suffering, it is also important to ensure that there is enough flexibility in the definition to prevent stagnation and encourage research into new probiotics.

The joint FAO/WHO working group released guidelines for the evaluation of probiotics in food and set out five headings to be appraised before bacteria can be classified as a probiotic (Martinez et al., 2015), which are outlined in Box 4.1.

It is also essential that the probiotic remains viable in the product’s final form and throughout the shelf life of the product, generally described as 28–30 days under refrigeration for yogurts (Vinderola et al., 2011). In addition, the probiotic in the product must retain its probiotic capabilities. However, studies have shown that the same strain may present different responses in vivo depending on its production or storage conditions. There are a number of factors that affect the functionality of a probiotic

**BOX 4.1 GUIDELINES FOR EVALUATION OF PROBIOTICS**

<table>
<thead>
<tr>
<th>Guideline</th>
<th>Brief Explanation</th>
</tr>
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<tbody>
<tr>
<td>Genus/Species/Strain</td>
<td>It is necessary to know the genus and species of the probiotic strain. There are possible exceptions to this requirement for certain bacteria such as <em>Streptococcus thermophilus</em> and <em>Lactobacillus bulgaricus</em>, which have a general ability to enhance lactose digestion in individuals with lactose intolerance. Thus individual strain identity is not crucial in this case.</td>
</tr>
<tr>
<td>In vitro tests to screen potential probiotics</td>
<td>These tests provide knowledge of strains and probiotic mechanisms. Each of these tests will require in vivo validation. In vitro tests include: bile acid resistance; resistance to gastric acidity; adherence to mucus and/or human epithelial cells and cell lines; antimicrobial activity against potential pathogens; ability to reduce pathogen adhesion; bile salt hydrolase activity; resistance to spermicides (for probiotics for vaginal use).</td>
</tr>
<tr>
<td>Safety considerations</td>
<td>Proof must be provided that a probiotic strain is safe and free of contamination in its delivery form.</td>
</tr>
<tr>
<td>In vivo studies in animals and humans</td>
<td>The principal outcome of efficacy studies on probiotics should be proven benefits in human and animal trials.</td>
</tr>
<tr>
<td>Health claims and labeling</td>
<td>In most countries probiotic foods are only allowed to display general health claims. The group recommends that specific health claims be allowed in cases where sufficient scientific evidence is available to back up such a claim. This would also prevent misleading information on labels where the probiotic has a quite specific function and is advertised under a general umbrella term.</td>
</tr>
</tbody>
</table>


bacterium: culture production, technological processes, shelf life, and biological barriers (Vinderola et al., 2011). Due to this the current plate count method to assess viability may not be a true representation of probiotic functionality. At any of the above stages, from production to consumption, a stress may cause the bacterial strain to lose certain functional attributes though the cell remains viable. Thus while the appropriate numbers of the probiotic are present at the end of shelf life, the probiotic effect may not take place. For example, the initial step in producing a probiotic strain for large-scale yogurt production involves its growth in suitable media. There are a number of variables associated with every media type, each of which could potentially alter the metabolism or growth of a strain. One study investigating the resistance of Lactobacillus rhamnosus to bile salts and gastric acidity revealed that the composition of the cryoprotectant influenced viability and gastric resistance of the final culture (Saarela et al., 2009). Such information is invaluable and as such there are a number of novel technologies being developed to allow the use of sensitive gut-specific probiotic microorganisms to be cultivated and incorporated into foods. These methods are aimed at maintaining the viability of the probiotic in the functional food as well as following transport through the digestive system (Ross et al., 2005). This represents an area that could allow for the use of a much wider variety of probiotics than those currently being studied.

Probiotic enumeration in yogurts remains a barrier to the industry as a whole. Standardization of the count method is desirable but as of yet has not been possible due to a number of complications. The probiotic bacteria used in yogurts are mainly derived from species closely related to the starter bacteria in terms of their metabolism. This means it is difficult to find a growth medium that favors the probiotic and yet not the starter cultures. In addition to this, as the starter is added and grows to high numbers, it is likely to outcompete the probiotic. Some products contain a combination of probiotic bacteria and if these are derived from the same species it is not possible to differentiate between them using the plate count technique (Sanders, 2008). While culture-independent techniques (e.g., nucleic acid amplification, fluorescent microscopy, MALDI-TOF mass spectrometry, etc.) offer alternative options for probiotic enumeration (Davis, 2014), such methods are likely to be time-consuming, require specialized expensive equipment and trained personnel. The development of inexpensive methods that offer better probiotic identification and enumeration in food products is an area of scientific research that should vastly aid the food industry.

4.5 PROBIOTICS USED IN YOGURTS

A range of probiotic yogurts is now commercially available and harbor probiotic strains such as Lactobacillus casei, Bifidobacterium bifidum, and L. rhamnosus GG (LGG) and claim to improve conditions such as constipation, antibiotic-associated diarrhea, eczema, etc. (Table 4.1). The best-studied probiotic lactic acid bacterium is LGG (Segers and Lebeer, 2014). Numerous research articles in peer-reviewed scientific journals investigating the health effects of this probiotic are available, and a number of potential benefits from consumption of LGG have been proposed (Douillard and de Vos, 2014). The strain has exhibited encouraging results in clinical trials for the treatment of diarrhea in patients from infants to the elderly (Szajewska and Kolodziej, 2015). Moreover, in a double blind, randomized, placebo-controlled trial involving renal patients who consumed a commercial yogurt containing LGG for 8 weeks, yogurt consumption was shown to clear vancomycin-resistant enterococci (VRE) in all patients (Manley et al., 2007). Interestingly, the LGG SpaCBA-SrtC pilus cluster shares 30%–40% sequence identity with the VRE pilus clusters (Kankainen et al., 2009) and provides the grounds for a
Table 4.1 Examples of Probiotic Yogurts Currently on the Market and Some of the Areas of Health They Claim to Improve

<table>
<thead>
<tr>
<th>Probiotic Strain</th>
<th>Yogurt Brand/Manufacturer</th>
<th>Target</th>
</tr>
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<tbody>
<tr>
<td><em>Lactobacillus bulgaricus</em></td>
<td>Yoplait, Stonyfield Farms, Dannon, Fage, Greek Gods Yogurt, La Yogurt, Voskos Greek Yogurt</td>
<td></td>
</tr>
<tr>
<td><em>Streptococcus thermophilus</em></td>
<td>Yoplait, Chobani, Stonyfield Farms, Dannon, Fage, Greek Gods Yogurt, La Yogurt, Voskos Greek Yogurt</td>
<td></td>
</tr>
<tr>
<td><em>Bifidobacterium bifidum</em></td>
<td>Chobani, Stonyfield Farms, Fage, Greek Gods Yogurt, La Yogurt, Voskos Greek Yogurt</td>
<td>AAD*</td>
</tr>
<tr>
<td><em>Lactobacillus casei</em></td>
<td>Chobani, Fage, Greek Gods Yogurt, Voskos Greek Yogurt</td>
<td>Respiratory tract infections</td>
</tr>
<tr>
<td><em>Lactobacillus rhamnosus</em></td>
<td>Stonyfield Farms</td>
<td>Diabetes, diarrhea, AAD, improve barrier function</td>
</tr>
<tr>
<td><em>Bifidobacterium animalis</em></td>
<td>Activia by Danone</td>
<td>Constipation</td>
</tr>
<tr>
<td><em>lactis</em> DN-173 010</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>L. casei</em> DN-114-001</td>
<td>DanActive by Danone</td>
<td>AAD, prevention of pediatric diarrhea, respiratory infections</td>
</tr>
<tr>
<td><em>L. casei</em> Shirota</td>
<td>Yakult</td>
<td>Constipation, <em>H. pylori</em></td>
</tr>
<tr>
<td><em>Bifidobacterium animalis</em></td>
<td>La Yogurt</td>
<td>Eczema</td>
</tr>
<tr>
<td><em>BB12</em></td>
<td>Vifit by Valio</td>
<td>Acute pediatric diarrhea, AAD</td>
</tr>
<tr>
<td><em>L. rhamnosus</em> GG</td>
<td></td>
<td>Acute pediatric diarrhea, cholesterol</td>
</tr>
<tr>
<td><em>Lactobacillus reuteri</em> DSM 17938</td>
<td>Protectis by BioGaia</td>
<td></td>
</tr>
</tbody>
</table>

*AAD, antibiotic-associated diarrhea.*

novel probiotic mechanism. Binding of *Enterococcus faecium* to mucus may be prevented by the presence of antibodies against LGG or SpaC, or the mucus binding protein SpaC itself. This experiment points at potential uses for a yogurt supplemented with the probiotic LGG or its pilins for prophylaxis and treatment of VRE infections. LGG has also been proven to reduce the prevalence of *Streptococcus mutans* in the oral cavity; *S. mutans* is a causative agent of dental caries (Nikawa et al., 2004).

LGG was discovered by Professors Gorbach and Goldin while they searched for a *Lactobacillus* strain to prove Metchnikoff’s theory that yogurt cultures could colonize the human intestine and benefit health. LGG is capable of adhering to the mucus layer of the intestinal tract, improving gut barrier function by preventing pathogenic bacteria from attaching. In addition, the strain has been shown to persist for longer in the host than closely related strains; indeed, orally administered LGG can be recovered from feces at least 1 week after administration and colonic biopsies have suggested that LGG can persist longer than this (Goldin et al., 1992). In a safety respect, LGG is consumed in high amounts in Finland and Sweden where the levels of reported *Lactobacillus* bacteremia have remained constant. LGG has been administered to high-risk patients such as pregnant women and HIV-infected patients with no detrimental side effects.
4.6 PREBIOTICS

While probiotic additions to yogurt introduce new beneficial bacteria, prebiotics in contrast act as a source of nutrients for the probiotics, and in this way prebiotics can be seen as a beneficial bacterial fertilizer. The prebiotic concept was introduced in the 1990s by Gibson and Roberfroid who defined prebiotics as “non-digestible 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 improves host health” (Roberfroid et al., 1993). As discussed herein in relation to probiotics, a consensus definition for prebiotics is equally important for the continued use of the term. This definition has retained its main features since its inception but has been revised on numerous occasions as the area of research and our understanding develop. In 2007, Roberfroid proposed an update of the definition as follows, “a prebiotic is a selectively fermented ingredient that allows specific changes, both in composition and/or activity in the gastrointestinal microflora that confers benefits upon host well-being and health” (Roberfroid, 2007); while this did not change the definition, the paper stated that only two dietary oligosaccharides fulfill the criteria for prebiotic classification, inulin and the galacto-oligosaccharides. In the same year, the FAO technical meeting on prebiotics defined the term as “a non-viable food component that confers a health benefit on the host associated with modulation of the microbiota.” This version no longer requires selectivity and is not limited to the gastrointestinal microbiota, and it also removes the need for a proven link between the prebiotic and altered microbiota/metabolism. The report also outlines the necessary qualifications for each part of the definition: (1) The prebiotic component may not be an organism or a drug. (2) Associated health benefit(s) must be measurable and not due to the absorption of the component or the component acting alone. (3) Modulation of the microbiota includes changes in composition or activities of the microbiota by the prebiotic in its deliverable form, and the prebiotic must induce these effects when consumed in the yogurt. This modulation could be due to a range of mechanisms including fermentation. In 2010, the latest definition was published following the sixth International Scientific Association for Probiotics and Prebiotics (ISAPP) meeting and is as follows, “Dietary prebiotic: a selectively fermented ingredient that results in specific changes in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefit(s) upon host health.” While highlighting dietary prebiotics that target the gastrointestinal tract, this definition is in disagreement with the FAO definition. The selective fermentation and focus on health brings the definition closer to the first definition. While redefining the term when new evidence emerges is important, it is crucial that a consensus is reached to avoid confusion among consumers or even lack of knowledge of the definition (Hutkins et al., 2016). ISAPP is currently organizing an expert panel to review the definition for prebiotics and reach a conclusion on where it stands based on current research (personal communication).

4.7 PREBIOTIC YOGURT

Prebiotic-supplemented yogurts have shown promising results in increasing probiotic counts and often the main sources of these prebiotics are fruit by-products. For example, pineapple waste powder (PWP) has recently been investigated as a potential prebiotic for use in yogurt manufacture (Sah et al., 2016b). The effect of PWP was evaluated on several probiotic strains including Lactobacillus acidophilus, Lactobacillus casei, Lactobacillus paracasei, as well as on the starter bacteria S. thermophilus and L. bulgaricus. PWP is rich in dietary fiber and was found to exhibit prebiotic effects on all strains with
the exception of *L. bulgaricus*. The proteolytic activities of each culture were found to be increased with the potential prebiotic addition. The yogurt itself also displayed increased antioxidant and antimutagenic activities compared to the nonsupplemented controls.

Studies have also examined the potential for prebiotic yogurts to directly treat illnesses. One such study indicated that a yogurt formulation of polydextrose, *Bifidobacterium lactis* HN019 and *L. acidophilus* NCFM positively impacted patients with constipation. The yogurt resulted in shortened colonic transit time and represents a possible treatment for constipation (*Magro et al.*, 2014).

### 4.8 SYNBIOTICS

Synbiotics refer to mixtures of prebiotics and probiotics together, where the prebiotic will improve the survival of the probiotic or other beneficial bacteria in the colon, which in turn confers health benefits on the host (*Macfarlane et al.*, 2008). However, this term should only be used when a true synergistic relationship between the probiotic and prebiotic exists in the deliverable food. Many food items containing both prebiotics and probiotics do not in fact conclusively produce a beneficial effect in all individuals (*Palaria et al.*, 2012).

### 4.9 SYNBIOTIC YOGURT

Daily consumption for 4 weeks of a yogurt supplemented with LGG and the prebiotic fructooligosaccharides (FOS; Actilight) in both healthy adults and elderly subjects suffering from constipation was found to regularize stool production in the elderly (*Granata et al.*, 2013). This is presumably due to the ability of the oligosaccharide to retain water in the fecal matrix, thus promoting regulation (*Roberfroid*, 2005).

A combination of probiotics, namely *Lactobacillus* spp. and *Lactococcus* spp. and the prebiotics palatinose, inulin, and α-cyclodextrin were used to create a symbiotic yogurt (*Pranckute et al.*, 2014). In this case, the prebiotic helped to regulate the growth of the probiotic species as well as to increase their antibacterial activity. This work highlights once again how the microbiology of a synbiotic yogurt could be used to beneficially affect the gut microbiome and confer health benefits on the host.

### 4.10 HEALTH-RELATED STUDIES INVOLVING PREBIOTICS, PROBIOTICS, AND SYNBIOTICS

When investigating the beneficial health attributes of yogurts supplemented with probiotics and prebiotics, it is necessary to appreciate that yogurt’s main role may be in the prevention (rather than the cure) of disease, where modulation of the gut microbiota may play a larger role in maintaining health rather than curing established diseases (*Maldonado Galdeano et al.*, 2015).

While some yogurts can help with the treatment of disease, the primary aim of research in this area focuses on yogurt as a method to lower the risk of becoming ill in the first place: prevention and protection (*German*, 2014). This requires a measure of individual health to prove a health claim/benefit was taking effect. The burden of proof is much harder in this scenario; it is easier to demonstrate that a
disease/infection is improving due to an intervention than it is to say the disease/infection never took hold because of the yogurt intervention. However, there have been great research efforts in recent years into the possibility of utilizing functional foods in a disease setting, with promising results for the future (Seale and Millar, 2013).

4.10.1 IRRITABLE BOWEL SYNDROME

It has been hypothesized that a disturbed gut microbiota may have a role in the development and symptoms associated with irritable bowel syndrome (IBS). Bearing this in mind, the use of probiotics/prebiotics could be used in an attempt to maintain a healthy gut and prevent the onset of such diseases (Lorea Baroja et al., 2007). The cause of IBS is not yet fully understood but increasing evidence suggests that it could develop following the perturbation of the gut microbiome. IBS patients have a less complex microbiota, which is associated with lower numbers of lactobacilli and bifidobacteria, both of which have antiinflammatory effects in the intestine. The administration of probiotics could help to normalize these levels and stabilize the gut microbiota. However, studies in this area report huge variation due to discrepancies between different trial methods and inadequate sample size. While probiotics as a whole seem to exert beneficial effects in IBS treatment, as of yet the strain, dose, treatment duration, or best formulation remains unknown due to lack of sufficient studies (Quigley, 2015).

In a study involving female volunteers suffering from IBS, an encapsulated form of the probiotic *Bifidobacterium infantis* 35624 was found to be significantly superior to placebo when ingested at a dose of $1 \times 10^8$ colony forming units (cfu)/mL for 4 weeks (Whorwell et al., 2006). Importantly, this study also investigated dose levels. The incorporation of this strain into a yogurt could potentially provide a method for reducing side effects associated with IBS without the need for medication.

The results from prebiotic trials are less abundant and often show conflicting results. This is likely due to different doses consumed; however; prebiotics have also been associated with exacerbating the symptoms of IBS, such as flatulence and abdominal pain. Currently there is insufficient evidence regarding synbiotic use against IBS.

4.10.2 LACTOSE INTOLERANCE

Lactose intolerance (or lactose malabsorption) can be described as an inability to adequately digest lactose; this is caused by a lack of small intestinal lactase activity. People who suffer from lactose intolerance experience gastrointestinal symptoms following the consumption of milk and milk products that contain high levels of lactose. The undigested lactose is fermented in the colon where it causes a range of effects from abdominal pain to diarrhea. Initially, it was believed that lactose-intolerant patients tolerated yogurt better than milk due to its lower lactose content. Subsequent studies revealed that the lactose in yogurt is digested by bacteria-derived lactase in the duodenum where the enzyme survives the acidic conditions of the stomach by being physically shielded in the bacterial cells and through the buffering capacity of the yogurt (Savaiano, 2014). Traditional starter cultures of *L. bulgaricus* and *S. thermophilus* act in a similar manner to an enzyme supplement by aiding the breakdown of lactose in maldigesters. Due to this ability to improve lactose digestion, both bacteria qualify as probiotics. *Bifidobacterium* spp. such as *Bifidobacterium longum* and *Bifidobacterium animalis* can also aid in lactose digestion.
4.10.3 ANTIBIOTIC-ASSOCIATED DIARRHEA

*Clostridium difficile*–associated diarrhea (CDAD) is often encountered in hospital settings where antibiotic treatment is routinely used (Vonberg et al., 2008). Antibiotic treatment greatly disrupts the intestinal microbiome allowing opportunistic pathogens to infect the host. This dysbiosis could be helped by the use of probiotics to help maintain the natural gut bacteria and aid in its reestablishment following the course of antibiotics. Bio-K+, a probiotic formulation of three *Lactobacillus* species, has been marketed in the United States since 1996 (https://www.biokplus.com/en_ca/products/c-difficile). This is sold as both a yogurt drink and in capsule form and it has been clinically proven that when Bio-K+ is taken in combination with antibiotics, it can reduce the risk of CDAD in hospitalized patients. It is composed of *L. acidophilus* CL1285, *L. casei* LBC80R, and *L. rhamnosus* CLR2, and the results of the study indicate that prevention of infection could be due to antimicrobial action and toxin neutralization molecules produced by the lactobacilli (Gao et al., 2010). It is recommended that probiotics, such as Actimel, be consumed while on a course of antibiotics in an attempt to maintain gut homeostasis (Williams et al., 2010). However, the European Food Safety Authority concluded that there is not enough cause-and-effect evidence for Actimel, a probiotic yogurt drink, to be unequivocally classed as effective. Antibiotic stewardship has been proven to be effective in lowering the number of *C. difficile* infections in health care settings (Hickson et al., 2007). Studies stating the contrary have also been published, but it has been indicated that this could be due in part to poor compliance.

Numerous other studies investigating the use of probiotics to treat or prevent CDAD infection have been completed with varying results, and so there is currently insufficient evidence to draw any exact conclusions (Auclair et al., 2015).

A new potential treatment against CDAD is the bacteriocin thuricin CD. This bacteriocin has been shown to be as effective as the currently prescribed antibiotics in the treatment of *C. difficile* (Rea et al., 2010). This particular study also revealed that the microbial diversity in the gut was not disturbed upon treatment with the bacteriocin, unlike with antibiotics, which cause collateral damage of massive disruptions in the microbiota (Rea et al., 2010). The narrow spectrum nature of thuricin CD removes this risk by targeting only the pathogen *C. difficile*. The use of bacteriocin-producing probiotics is a new and exciting area for fighting against antibiotic resistant pathogens, and to prevent the use of broad-spectrum antibiotics on known infections.

4.10.4 RESPIRATORY TRACT INFECTIONS

Respiratory tract infections (RTIs) are common to a high proportion of the public, especially children, and while they are normally self-limiting, they can lead to complications. Increased antibiotic resistance in RTIs is a global issue. Studies and trials have been performed to see if probiotics/prebiotics could be used to reduce susceptibility to these infections. In a systematic review of the effectiveness of different probiotic strains to prevent RTIs, the results of 14 randomized controlled trials were examined (Vouloumanou et al., 2009). This review concluded that while probiotics did not reduce the incidence of infection, symptom severity and duration were reduced.

4.10.5 CARDIOVASCULAR DISEASE

Cardiovascular disease (CVD) is the leading cause of death worldwide, with numbers rising each year (WHO factsheet). Hypercholesterolemia is the leading risk factor in CVD incidence, thus intervention is
important to lower cholesterol levels in high-risk individuals. Pharmacological methods do exist, but it is increasingly more common for patients to wish to use a nonmedicinal, more natural method to lower cholesterol, due to adverse side effects or personal preference. Probiotic yogurt formulations have been investigated to reduce cholesterol levels (Ataie-Jafari et al., 2009). One study using \textit{L. acidophilus} and \textit{B. lactis} supplemented yogurt examined blood lipid levels following consumption of the probiotic yogurt over a course of 6 weeks. It was found that those consuming the probiotic yogurt were shown to have significantly lower cholesterol levels than that of the control group, which consumed traditional yogurt. This indicates a cholesterol-lowering effect with consumption of the probiotic yogurt in mildly to moderately hypercholesterolemic participants. Ingestion of \textit{Lactobacillus mucosae} DPC 6426 has also been proven to reduce serum cholesterol associated with changes of the gut microbiota in a murine model (London et al., 2014). This strain has since been investigated as an adjunct culture for yogurt manufacture and was shown to remain viable throughout storage, indicating yogurt as a potential delivery vehicle (London et al., 2015).

Another probiotic yogurt study on cholesterol involved microencapsulated \textit{Lactobacillus reuteri} NCIMB 30242, a bile sat hydrolase-active strain, taken twice daily for a 6-week period (Jones et al., 2012). The results of this study indicate that the probiotic yogurt was safe and effective in lowering total cholesterol in hypercholesterolemic adults over the test period. This formulation compares well with other cholesterol-lowering food ingredients. However, the time to maximal effect may be longer than other therapies, though it is indicated in the report that a repeat trial in which the length of study would be extended could possibly reach a higher therapeutic level than the endpoint of this trial.

Prebiotic studies have also taken place on possible cholesterol lowering effects but the results of these trials have not been consistent. One study reported that oral administration of 7 g of inulin over 4 weeks reduced total cholesterol levels in obese and hypercholesterolemic subjects (Balcazar-Munoz et al., 2003).

### 4.10.6 OSTEOPOROSIS

Osteoporosis is a disease that weakens bones to the point that they are prone to breaks and fractures. Research into altering the human microbiome to help regulate bone mass is relatively new. There is evidence to support the theory that low-grade inflammation affects bone mass, and this has led to the hypothesis that the gut microbiota’s influence on the immune system could be used as a novel target for bone mass regulation (Ejtahed et al., 2016).

Several hypotheses have been proposed on the potential mechanisms of the prebiotic FOS in increasing calcium absorption and retention. One proposed mechanism is that bacterial species ferment FOS, which increases the presence of short-chain fatty acids and lactic acid. This in turn causes the pH in the lumen to drop and insoluble Ca phosphate is dissolved in the now acidic environment. This will increase absorption through passive diffusion. This field is relatively new, but increased interest in the area could produce some interesting results on the impact of the gut microbiome on bone health and open the door for bone health–targeted prebiotic food products such as yogurts.

### 4.10.7 HELICOBACTER PYLORI INFECTION

\textit{Helicobacter pylori} is a gram-negative pathogen that is acid tolerant, microaerophilic and is found in the stomach and duodenum. It is one of the leading etiological agents of peptic ulcer disease as well as gastritis and in some cases can lead to the development of gastric cancer. Lactobacilli secrete lactic acid, which is inhibitory toward \textit{H. pylori}. Animal studies have shown that oral administration of a
range of \textit{Lactobacillus} species reduces the viability of the pathogen and its ability to adhere to human mucosal cells. These studies indicate that probiotics could be a viable solution to help reduce \textit{H. pylori} incidences and gastric colonization. The rise in antibiotic-resistant strains makes the search for new preventative therapies even more important. In a randomized, double-blind study, Francavilla et al. (2014) investigated the effects of a probiotic yogurt (\textit{Lactobacillus reuteri} DSM 17938 and \textit{L. reuteri} PTA 6475) versus a placebo to inhibit \textit{H. pylori}. The results of this study showed that the probiotic combination alone is enough to inhibit the growth of the pathogen, and when taken in combination with eradication therapy, a significant reduction in antibiotic-associated side effects was reported. In contrast, a study investigating the effects of probiotic treatment for peptic ulcers, using \textit{L. acidophilus}, \textit{L. rhamnosus}, \textit{B. bifidum} and \textit{Streptococcus faecium}, did not show any beneficial results in increasing efficacy of antibiotic treatment or reduction in adverse side effects compared with the placebo (Navarro-Rodriguez et al., 2013). A meta-analysis of clinical studies that used multistrain probiotics as adjunct therapy for eradication of \textit{H. pylori} and prevention of side effects concluded that some probiotic combinations can be effective but not all formulations were successful (McFarland et al., 2016). The effects of these successful formulations can range from reduced adverse side effects to reduced antibiotic-associated diarrhea or eradication, all when taken with the standard eradication therapy. Further research into specific strains is required for research in this area to advance.

4.10.8 ALLERGIC DISEASE

Recent studies have suggested a rational belief that the gut microbiome plays an important role in the rise of atopic allergies; this is due to its influence on sensitization and ability to modulate immunologic and inflammatory systemic responses (Capurso, 2001). The hygiene hypothesis has a role in the rationale for this approach; however, there are inherent complexities involved. Changes in early life exposure to the microbial world cause a change in the microbiome, and epidemiological studies have shown that early life exposure to pathogenic and nonpathogenic microbial products does have an influence on allergic responses. To combat this, probiotics, prebiotics, and synbiotics are being studied.

The differences recorded in the microbial compositions of allergic and nonallergic children suggest that the colonization period postbirth could be a target to prevent allergic diseases. Kalliomaki et al. (2001) reported a 50\% drop in the development of infantile eczema through the administration of probiotics to the pregnant mother and during the early postnatal period. The same group reported that LGG given to mothers and infants for 6 months after birth significantly reduced the development of eczema and asthma when compared to the control group (Kalliomaki et al., 2001). However, contradictory results have also been reported, which state LGG had no effect on incidence or severity and in fact increased incidence of wheezing episodes were associated with the probiotic group (Kopp et al., 2008).

Synbiotic studies have shown promising results in atopic patients. A combination of \textit{L. salivarius} and the prebiotic FOS was shown to be superior to FOS alone for treating children of 2 and 14 years of age with moderate to severe atopic dermatitis (Wu et al., 2012). While the mentioned studies do not involve a yogurt preparation, they do indicate the possibility of using probiotic/prebiotic/synbiotics to treat allergic diseases, which could in the future result in functional food preparations.

The World Allergy Organization-McMaster University Guidelines for Allergic Disease Prevention (GLAD-P) suggests using prebiotics in non-exclusively breast-fed infants but not in breast-fed infants. This recommendation is conditional, because although the panel determined that using prebiotics in infants provides a net benefit, this is with very low certainty due to the nature of the studies completed
(Cuello-Garcia et al., 2016). No recommendations were made for pregnant women or breast-feeding mothers as there are currently not enough evidence-based studies on the benefit or adverse effects of prebiotic supplementation in pregnant women to elucidate recommendations on whether or not prebiotics given during pregnancy will reduce the likelihood of a child developing an allergy. The European Academy of Allergy and Anaphylaxis Guidelines and the other allergy agencies in both Europe and North America concluded that although there is some evidence base for prebiotics having a beneficial effect, currently there is insufficient evidence to warrant a change in practice.

4.11 CONCLUSIONS AND FUTURE PERSPECTIVES

While yogurt has long been associated with promoting better health, our understanding of the scientific mechanisms behind this concept have deepened, as we learn more about the importance of the gut microbiota to overall health. Its native cultures, *L. bulgaricus* and *S. thermophilus*, have been linked to several health benefits including improved lactose digestion in sufferers of lactose intolerance. Yet the beneficial attributes of yogurt can be further enhanced through the addition of probiotics and prebiotics, where it provides an ideal protective medium that is already accepted by consumers. In this regard, it could be touted as the “model” functional food. One of the greatest challenges to the success of functional yogurts is the same as that of the probiotic or prebiotic itself, i.e., the need to provide scientifically sound evidence of the proposed beneficial effects. Functional yogurts are more likely to serve as a prophylactic aid rather than a therapeutic treatment and, in this regard, the proposed health benefits can be difficult to quantify in any metric fashion. Once the health attribute has been confirmed through in vitro and in vivo studies and clinical trials, it is important that the scientific community, regulatory agencies, and industry leaders come together to agree on the exact specifications required for the health claim to be permitted. This is made difficult due to the varying desired outcomes; the scientific researchers desire long-term competitive funding on a project, the industry is searching for profitable yogurt products, and the regulatory agency desires transparency and safety for the consumer. Bringing all of these goals together will be required for this field to thrive. Moreover, the lack of regulation and legal definitions is beginning to hinder the industry as well as the fact that there are no standard tests available currently for the enumeration of probiotics and prebiotics, which requires attention.

Another consideration, which is no less important from a consumer point of view, is that prebiotics and probiotic cultures can impact yogurt rheology particularly through EPS production in the case of probiotics (Hekmat and Reid, 2006), while prebiotics have an impact on bacterial growth and so potentially alter the structure of the yogurt matrix. Indeed, prebiotics are capable of being used as a fat substitute, bulking agent, low-calorie sweetener, and texture modifier when added to yogurt (Allgeyer et al., 2010). Large changes in rheology are not generally received well by consumers, therefore the sensory properties of the final product are just as important as the health claims from a business point of view. This can be overcome by using suitable combinations of strains and generating sensory analysis on the final products to ensure that they meet consumer demands.

Furthermore, it is essential that the probiotic mechanisms of the individual strains used are understood and that these mechanisms are translatable to the mammalian host following processing and formulation of the final product. This can only be achieved through intelligently designed randomized, double-blinded clinical trials ensuring that the final product does what it says on the pot enabling the production of specific health-targeted foods that will ultimately aid a healthier lifestyle for its consumer.
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REFERENCES


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**FURTHER READING**

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5 OTHER FERMENTED DAIRY PRODUCTS: KEFIR AND KOUMISS

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5.1 INTRODUCTION

The preservation of food by fermentation is one of the oldest methods known to humankind. Fermented milk has been developed to preserve milk against spoilage. It is likely that the origin of these products was the Middle East and the Balkans, and it is safe to assume that these products may date to more than 10,000 years ago. Their evolution through the ages has progressed from home manufacture by artisanal processes, using a small portion of a previous batch as starter, to large-scale production (Litopoulou-Tzanetaki and Tzanetakis, 2014). Fermented milk products have been classified into three groups: (1) lactic fermentation products (mesophilic, thermophilic, and probiotic), (2) yeast-lactic fermentation products, and (3) mold-lactic fermentation products. A typical example is lactic acid fermentation, which is widely used for the preparation of several fermented milk products, such as dahi (curd), yogurt, acidophilus milk, shrikhand, and various varieties of cheeses. The role of fermented milk in human nutrition is well documented. Man knew the virtues of these fermented foods, even during the early days of civilization. In earlier days, these foods were produced by natural fermentation with the main objective of preserving milk. In Europe, Asia, and Africa, sour milk was known to be more stable than fresh milk. It preserved the high-quality nutrients present in milk in a relatively more stable form (Shah, 2014). The lactic-yeast fermentation products that originated from Central Asia between the Caucasus Mountains and Mongolia are popular in many countries, including the former Soviet Union, Poland, the Czech Republic, Slovakia, Hungary, Bulgaria, Turkey, and some Scandinavian countries. The well-known examples of this class of fermented milk product are kefir, koumiss, and yeast-acidophilus milk. Kefir and, to a lesser extent, koumiss, have economic importance (Özer and Kirmaci, 2014). Kefir and koumiss are the fermented dairy products believed to have originated in central Asian and eastern European regions. Kefir means “feel good” in Turkish and is considered a mixed lactic acid and ethanol fermented beverage. It is also manufactured under a variety of names including kephir, kiaphur, kefer, kefyr, kephir, kefer, kiaphur, kefer, knapon, kepi, and kippi, with artisanal production of kefir occurring in countries as widespread as Argentina, Taiwan, Portugal, Turkey, and France (Farnworth, 2005). Kefir is the most popular fermented milk product across all areas of Russia, Belarus, Bosnia and Herzegovina, Ukraine, Hungary, Romania, Poland, Norway, Sweden, Finland, Latvia, Estonia, and Lithuania. Kefir also occupies an important place in the human diet in other parts of the world including Southwest Asia, North America, Japan, the Middle East, and North Africa due to its nutritional and therapeutic properties (Shah, 2014).
Koumiss (also known as kumys or kumiss) is another fermented milk product of Russian and East European origin and is produced by a mixed yeast-lactic fermentation. Koumiss is a unique lactic acid–alcohol fermented milk drink that originated in central Asia. The name is derived from the Kumanese, who survived until 1235 as a Kumane River tribe on the central Asian steppes (Akuzawa et al., 2011). Similar to kefir, koumiss has a long tradition dating some 25 centuries back when the Scythian tribes enjoyed benefits of this refreshing drink (Koroleva, 1991). It is widely consumed in western and central Asia (e.g., Mongolia, Kazakhstan, Kyrgyzstan) and Russia for its nutritive and therapeutic properties (Uniacke-Lowe, 2011). Traditionally, koumiss is manufactured from mare’s (horse’s) milk. However, for industrial-scale production of koumiss, cow’s milk is used as mare’s milk remains a very limited commodity. Cow’s milk is richer in fat and protein but lower in lactose than mare’s milk. Hence, if cow’s milk is used, the milk is fortified by adding sucrose or modified whey to better approximate the composition of mare’s milk and to allow comparable fermentation.

Kefir and Koumiss both are yeast-lactic fermented milk products. Both have their own typical product characteristics and manufacturing methods, which will be discussed in detail later. Many reviews and book chapters are available on various aspects of kefir (Özer and Özer, 2000; Farnworth and Mainville, 2003; Wszolek et al., 2006; Rattray and O’Connell, 2011; Özer and Kirmaci, 2014; Litopoulou-Tzanetaki and Tzanetakis, 2014) and koumiss (Özer and Özer, 2000; Wszolek et al., 2006; Uniacke-Lowe, 2011; Özer and Kirmaci, 2014; Litopoulou-Tzanetaki and Tzanetakis, 2014; Dhewa et al., 2015). Besides general descriptions and product characteristics, this chapter attempts to highlight recent technological advances in kefir and koumiss production.

5.2 PRODUCT CHARACTERISTICS OF KEFIR

Kefir, an acid-alcohol fermented milk product, originally made in the Balkans, Eastern Europe, and the Caucasus, is traditionally produced by inoculating milk with grains of kefir (Ismaiel et al., 2011). The word *kefir* is derived from the Turkish *kef*, which means pleasant taste (Kurmann et al., 1992). Kefir is a self-carbonated, refreshing fermented milk product with a slight acidic taste made from kefir grains, a complex and specific mixture of bacteria and yeasts held together by a polysaccharide matrix (Shah, 2014). Kefir is not a curdled product and is produced by the addition of kefir grains to fresh milk (Loretan et al., 2003). A wide community of microorganisms present in kefir grains is lodged in the grains in a polysaccharide kefiran matrix. Traditionally, the fermentation of milk was carried out 24h at room temperature in goatskins, clay pots, or wooden buckets in which milk from cows, goats, sheep, camels, or buffalo was used as the fermentation substrate. Other substrates for kefir production include soymilk, fruit juices, sugar, or molasses. In goatskin bags, the content was tied off in one corner of the leather bag (where most of the grains were retained), and the kefir separated from the grains by pouring the beverage off. This produced a foaming drink, creamy in consistency and texture with the alcohol content of approximately 0.08%–2.0% (Anfiteatros, 2004). During 24h fermentation, kefir grains change the milk into a thick, astringent tasting beverage. During cold weather, leather bags were placed in the sun during the day or hung near a fireplace during the night. It was also a custom to hang the bag near a doorway, where visitors would give the bag a gentle rock as they passed by (Koroleva, 1988). The fermented milk product is characterized by a distinctive yeast-like flavor and a fizzy or sparkling mouthfeel. In fact, kefir has a highly complex flavor because the kefir grains used in its manufacture have a highly diverse and complex microbiota. The principal fermentation products in kefir are lactate,
ethanol, and carbon dioxide, while other minor fermentation products include diacetyl, acetaldehyde, free amino acids, and, in some cases, acetate (Rattray and O’Connell, 2011).

Kefir was also regularly subjected to secondary fermentation, during which a mixture of fresh kefir, fresh milk, and the root of Snow Rose (Rhododendron caucasicum) was poured into wooden barrels or clay crosks (Koroleva, 1988; Anfiteatros, 2004). The container was then sealed airtight and the content fermented for some days. This produced a highly carbonated beverage, with possibly a slightly higher alcohol content (Anfiteatros, 2004).

The composition of kefir varies with the type of milk employed. A typical kefir contains 80%–90% moisture, 0.2% lipid, 3.0% protein, 6.0% sugar, and 0.7% ash and approximately 1.0% lactic acid and alcohol. Kefir also contains 1.98 g/L of carbon dioxide (Shah, 2014). According to Kosikowski and Mistry (1999), the typical composition of kefir is 1.5% fat, 10.5% total solids, 3.5% protein, 0.7% ash, and the pH is 4.6. Approximately 0.9% of total weight of kefir is represented by its microflora. Bacterial contents vary from $6.4 \times 10^4$ to $8.5 \times 10^8$ cfu/g and yeasts from $1.5 \times 10^5$ to $3.7 \times 10^8$ cfu/g. Kefir also contains $10^6$ cfu/g of acetic acid bacteria after fermentation (Shah, 2014). Lactic acid bacteria identified in kefir are Lactobacillus, Lactococcus, Leuconostoc, Lactobacillus delbrueckii ssp. bulgaricus, Lactobacillus kefiri, Lactobacillus kefirgranum, Lactobacillus acidophilus, Enterococcus faecium, Enterococcus faecalis, Streptococcus thermophilus, and Lactococcus lactis ssp. cremoris are also reported to be present. Identified yeasts include Kluyveromyces marxianus, Kluyveromyces lactis, Candida kefiri, Saccharomyces cerevisiae, Saccharomyces kefir, Saccharomyces unisporus, Zygosaccharomyces rouxii, Torulaspora delbrus, Torulaspora delbrueckii, and Debarymyces hansenii.

### 5.2.1 Kefir Grains

Kefir grains are a combination of bacteria and yeasts in a matrix of proteins, lipids, and sugars. This symbiotic matrix forms grains that resemble cauliflower. Kefir grains range in size from 0.3 to 2.0 cm or more in diameter and are characterized by forming an irregular, folded, or uneven surface; the grains resemble cauliflower florets in shape and color (Fig. 5.3). They are elastic and white or slightly yellow in color and have a characteristic smell (Wszolek et al., 2006). Kefir grains contain 86.3% moisture, 4.5% protein, 1.2% ash, and 0.03% fat. The dry mass of the fresh grains amounts to 10–16 g/100 g, which consists of about 30 g/100 g protein and 25–50 g/100 g carbohydrate (Libudzisz and Piatkiewicz, 1990). The chemical composition (g/100 g) of kefir grains originating from Russia, Yugoslavia, and Bulgaria contained approximately moisture 90, protein 3.2, fat 0.3, nonprotein soluble nitrogen 5.8, and ash 0.7 (Shah, 2014). Kefir grains are rich in polysaccharides, in which bacteria including L. kefiri, L. kefirgranum, Leuconostoc spp., Lactococcus spp., and Lactobacillus ssp. and yeasts such as S. kefiri, C. kefiri, and Torula spp. are embedded. Kefiran is a heteropolymer of glucose and galactose. Kefir grains contain a water-soluble polysaccharide known as kefiran that imparts a ropelike texture and feeling in one’s mouth (Shah, 2014). Kefir grain is composed of a diverse spectrum of species and genera including lactic acid bacteria (Lactobacillus, Lactococcus, Leuconostoc), yeasts (Kluyveromyces, Candida, Saccharomyces, and Pichia) and sometimes acetic acid bacteria (Acetobacter) in a symbiotic association. The most common lactobacilli isolated from kefir grains are L. kefiri, Lactobacillus kefirgranum, Lactobacillus parakefiri, L. delbrueckii, L. acidophilus, Lactobacillus brevis, Lactobacillus helveticus, Lactobacillus casei, Lactobacillus paracasei, Lactobacillus fermentum, Lactobacillus plantarum, and Lactobacillus gasseri (Angulo et al., 1993; Marshall, 1993; Takizawa...
Kefir-specific yeasts play a key role in the formation of flavor and aroma. They are represented by the species *K. marxianus*, *K. lactis*, *S. cerevisiae*, *T. delbrueckii*, *C. kefir*, *Pichia fermentans*, *Kazachstania unispora*, and *Kazachstania exigua* (Angulo et al., 1993; Marshall, 1993; Garrote et al., 2001; Simova et al., 2002; Zhou et al., 2009; Vardjan et al., 2013). The flora is complex and highly variable community of microorganisms. This microflora is embedded in a heteropolysaccharide matrix (kefiran) composed of equal amounts of glucose and galactose (Rea et al., 1996). Kefir grains have a complex microbiological composition, and they consist of a blend of lactic acid bacteria ~83%–90%, yeasts ~10%–17%, acetic acid bacteria, and possibly a mold (Wszolek et al., 2006). According to Polish Standards (Anonymous, 2002), the microscopic observation of the grains should consist of 80% *lactobacilli*, 12% yeasts, and 8% *lactococci*.

Wittthuhn et al. (2004) indicated that kefir grain microbiota strongly depends on the origin of the grains, the local conditions of culture, and the storage and manipulation processes. The growth and survival of individual strains in kefir grains are dependent on the presence of others; because of this, kefir grains are a good example of symbiosis. When the various microorganisms are separated as pure cultures, they do not grow in milk or have decreased biochemical activity. The growth of several bacteria isolated from kefir grains was improved when yeast extract was added to growth medium, indicating that the yeasts found in kefir grains were essential to maintain the integrity and viability of the microbiota population (Vardjan et al., 2013).

Kefir cannot be produced from scratch, as kefir grains can only grow from preexisting grains, but grains grow during fermentation and additional grains are produced. Kefir grain production is based on continuous cultivation in milk, and this results in an increase in biomass of 5%–7% per day. A combination of factors influences the biomass increase of the kefir grains including temperature (optimum temperature is 25°C), pH, addition of fresh washing of grains, renewal of milk, and presence of nutrients. The grains are insoluble in water and are resistant to enzymes and grains swell on soaking with water. Bacteria and yeasts grow symbiotically (Shah, 2014). Kefir grains have a specific structure and biological function. When the grains are seeded in milk, they grow and pass their properties to the following generation(s) of newly formed grains (Guzel-Seydim et al., 2000; Saloff-Coste, 2002; Simova et al., 2002). Furthermore, the microflora of kefir grains is remarkably stable, retaining its activity for years if preserved and incubated under appropriate cultural and physiological conditions (Wszolek et al., 2006).

In Russia, a mother culture is prepared by carrying out traditional kefir fermentation and straining the grains. Kefir grains can be reused several times provided the grains are recovered, dried, and stored under sanitary conditions. Once the milk is fermented to kefir, agitation of the curd causes migration of the grains to the surface and grains are strained out, rinsed in chilled water, dried in an oven or at room temperature (36–48 h), and stored in aluminum foil. Kefir grains are kept cool and dry until reused. Dry grains are stored for 12–18 months (Shah, 2014).

Koroleva (1988) cautioned that any attempt to replace kefir grains by a mixture of pure microorganisms would not be effective because the unique composition of kefir grains that has evolved over time cannot be replicated or replaced. Any symbiotic relationship between bacteria and yeasts that has developed over time is special; but for large-scale production of kefir with kefir grains, it is important that the microbiota of kefir grains should be stable and does not change over time. Only in this way can we be sure of a product of the required and consistent quality that fulfills the criteria for traditional kefir. Kefir grains serve as basic inocula in traditional kefir production, but their complex microbiological association makes it difficult to obtain defined and constant kefir starter culture appropriate for industrial kefir production of conventional properties (Robinson et al., 2002). Therefore, in recent years, attention has been focused on producing kefir from a mixture of pure and defined cultures. This enables
better control over the microorganisms involved and greater ease of production, but the properties of the final product significantly differ from kefir fermented with kefir grains (Marshall, 1993; Özer and Özer, 2000; Beshkova et al., 2002). However, despite the intensive research carried out to produce kefir grains from pure or mixed cultures normally present in the grains, no successful results have been reported (Koroleva, 1975; Hirota and Kikuchi, 1976; Liu and Moon, 1983; Libudzisz and Piatkiewicz, 1990). This failure can probably be ascribed to the fact that very little is known about the mechanism of grain formation (Schoevers and Britz, 2003). Detecting and identifying the microbial composition of kefir grains and kefir with a rapid method is often important for quality control of this product. Moreover, complete information regarding microbiota composition can enlighten the mechanisms and interactions involved in the production of several bioactive materials, particularly those responsible for kefir grain formation (Vardjan et al., 2013).

5.2.2 STRUCTURE OF KEFIR GRAINS

Naked-eye examination of kefir grains (Fig. 5.3) showed that kefir grains resemble small cauliflower florets that vary in size from 3 to 30 mm in diameter, are lobed, irregularly shaped, white to yellow-white in color, and have a slimy but firm texture (Garrote et al., 2001). When viewed with the naked eye, the exterior surfaces of the kefir grains look smooth and shiny. However, under scanning electron microscope, the grain surfaces were revealed to be very rough. Kefir grains have a varying and complex microbial composition that includes species of yeasts, lactic acid bacteria (LAB), acetic acid bacteria, and mycelial fungi. Lactobacilli are present as the largest portion (65%–80%) of the microbial population (Wouters et al., 2002), with lactococci and yeasts making up the remaining portion of the microbes present in the kefir grain (Witthuhn et al., 2005). In kefir grains both the bacteria and yeast are surrounded by a polysaccharide matrix, called kefiran, which is a water-soluble branched glucogalactan (Lee et al., 2007). Fig. 5.4 clearly demonstrates that the microflora is embedded in a resilient polysaccharide matrix composed of glucose and galactose that has been given the name kefiran (La Rivière et al., 1967). Nothing is known, however, of the mechanism of grain formation, and attempts at making kefir grains from pure or crude cultures have not been successful (Koroleva, 1975; Hirota and Kikuchi, 1976). Various workers have examined the structure of kefir grains and distribution of microbiota using light microscopy, electron microscopy (Marshall et al., 1984; Guzel-Seydim et al., 2005; Chen et al., 2005), and Magalhães et al. (2010) confocal laser scanning microscopy (CLSM). Light and electron microscopy of kefir grains clearly demonstrated that the mixed microflora is not indiscriminately intermingled but has a particular arrangement, and electron microscopy revealed a matrix composed largely of fibrillar carbohydrate in which the microflora is enmeshed (Fig. 5.4) and that appears essential for grain formation and integrity (Marshall et al., 1984). Transmission electron microscopy showed more details of their structure (Fig. 5.5). Most of the earlier researchers (Bottazzi and Bianchi, 1980; Tamime, 1983; Toba et al., 1990; Mukai et al., 1992; Lin et al., 1999) reported that yeast predominated at the center part of the granule while the exterior of the grain had mostly lactobacilli. However, Guzel-Seydim et al. (2005) reported that relatively fewer yeasts were observed in the sub-middle portion compared to the outer portion of the kefir grains. Kefir grains had a spongy fibrillar structure that was branched and interconnected. Fibrillar material increased progressively toward the interior portions of the grain but yeasts were not evident in this part of the kefir grains. This is perhaps because yeast is aerobic and more likely to colonize the kefir grain surface. Observations indicated that yeasts became less predominant further inside the grain. Lactobacilli,
yeast, and fibrillar material were observed at ×5000 and ×10,000 on the exterior portion of the grain (Figs. 5.6 and 5.7). The fibrillar material was most probably the polysaccharide kefiran. Three different types of lactobacilli (short, long, and curved) were observed embedded in the grain along with yeast cells. Magalhães et al. (2010) studied the kefir grains under CLSM, and calcofluor white (blue) fluorescent probe was used to highlight yeast cells in the microbial biomass. Blue-stained regions were found as smaller portions randomly distributed among the grain’s surface. A similar distribution pattern was observed in the internal surface of the grains, with macroclusters of yeasts distributed within the grain’s matrix, essentially composed of polysaccharides and bacteria. Use of the confocal system allowed for the determination that the wall of the kefir grain appears to be a nearly solid mass of bacterial cells, organized in a linear fashion with defined edges.

5.2.3 PRODUCTION OF KEFIR

The origins and production of kefir are lost to memory, and, traditionally, the method of producing kefir and kefir grains was a closely guarded secret. Today, kefir or kefir-like beverages are made across the globe, with reports of kefir making in Ireland, Spain, Turkey, Malaysia, Indonesia, Tibet, and North and South America. However, kefir remains most popular in Eastern Europe, where it is generally made from cow’s milk (Rattray and O’Connell, 2011). By and large, kefir is produced by adding either a starter culture called kefir grains directly or a percolate of the grains to milk. Kefir has variously been described as a “dairy champagne,” “the champagne of cultured dairy products,” and “yogurt of the 21st century” (Ouwehand and Salminen, 1998). Kefir differs from other fermented milk products in its starter, which exists in the form of grains (Simova et al., 2002).

The traditional method of kefir making involved direct addition of kefir grains (2%–10% m/v) to milk that has been pasteurized and cooled to 20–25°C. The milk and grains are then subjected to 24 h of fermentation at room temperature. After this, grains are removed by filtration and the beverage is ready for consumption (Shah, 2014). Fermenting for a short period produces a milder, sweeter tasting kefir, while a long fermentation produces a sour beverage (Anfiteatros, 2004).

A second method, known as the “Russian method,” involves two fermentations and is used for the production of kefir on a large scale (Fig. 5.2). The first fermentation allows for the preparation of the starter culture and is achieved by incubating milk with grains (2%–3% m/v). The grains are then removed by filtration and the mixture is added to milk (1%–3%) as starter culture. This milk is then subjected to a second fermentation of 12–18 h (Shah, 2014). Although these methods have been used for many years, traditional methods produce only small volumes of kefir, and require several steps, which increase the chances of contamination. The shelf life of traditional kefir is very short, usually less than 3 days, but if kept in glass bottles the beverage can be kept for 8–10 days at 4°C storage temperature (Koroleva, 1988). Kefir produced with kefir grains can vary substantially due to the diverse microbiological profile of the kefir grains. Also, variation in the fermentation process can result in significant changes in flavor and texture. Kefir production using kefir grains includes a stage called ripening, which occurs in the retail pack, where the kefir is held at 8–10°C for up to 12 h, whereupon it is chilled to refrigeration temperatures and is then ready for consumption. The purpose of this ripening stage is to allow for the correct growth of yeast and bacteria within the kefir. Omission of this step is linked to atypical flavor development (Rattray and O’Connell, 2011). Another method for kefir production at the commercial level is through the application of commercial starter cultures that are directly inoculated into the milk (Fig. 5.1). Use of commercial direct-to-vat cultures
simplifies the commercial production of kefir, and with careful selection of yeasts and bacterial species, it is possible to produce a product that has an acceptable kefir flavor and very good keeping qualities. Kefir made this way can have a shelf life of up to 28 days, as compared to kefir produced with kefir grains, which has a shelf life of 3–12 days (Rattray and O’Connell, 2011). However, this type of kefir lacks the microbial diversity of kefir made with grains and thus may not have the same therapeutic and probiotic characteristics.

FIGURE 5.1 Flow diagram for kefir production using commercial direct-to-vat cultures.

5.2.4 COMPOSITION AND SENSORY CHARACTERISTICS OF KEFIR

Kefir is a self-carbonated beverage that owes its distinctive flavor to a mixture of lactic acid, ethanol, carbon dioxide, and other flavor products, such as acetaldehyde and acetone. This unique flavor is the result of the symbiotic metabolic activities of a number of bacterial and yeast species, which include both proteolytic and lipolytic degradation of milk constituents (Vedamuthu, 1977; Choi and
FIGURE 5.3

Naked eye view of kefir grains.


FIGURE 5.4

Kefir grain stained with toluidine blue–convoluted side × 4100.

The sensory characteristics of kefir can be described as follows: (1) the color is white or yellowish; (2) the aroma is balanced and yeasty; (3) the taste is acidic, but pleasant and refreshing; and (4) the texture is rather thick, but not gluey, with an elastic consistency (Wszolek et al., 2006).

Ng-Kwai-Hang, 2003; Chen et al., 2005).
The composition (chemical, organoleptic, and microbiological characteristics) of the final product depends on the type of milk used, the source of grains, the preparation of mother culture (often produced by sieving the coarsely grains and using the percolate), the length of fermentation, the inclusion of cooling step, and the inclusion of a maturation step.

The chemical composition of kefir provides a useful indication of the nutritional value of the product. According to Halle et al. (1994) and Bottazzi et al. (1994), a typical compositional analysis (g/100 g) of kefir consists of protein 3.0–3.4, fat 1.5, and lactose 2.0–3.5 (after the fermentation stage). However, the lactic acid content may range between 0.6 and 1.0 mL/100 mL lactic acid, and the alcohol level may be 0.0–0.1 g/100 mL in kefir made with a starter culture fermentate, or 0.03–1.8 g/100 mL in kefir made with kefir grains (Wszolek et al., 2006). Detailed chemical compositions of different types of kefir are given in Table 5.1. Kefir is considered a probiotic resource, and in addition to its nutritional value, a number of health claims have been made regarding it as having immuno-modulatory, antineoplastic, and prodigestive effects (Yang et al., 2008). The taste of unflavored kefir has been described as “yeasty,” and the terms “prickling” and “sparkling” have been used to describe the mouthfeel of kefir caused by the liberation of trapped CO$_2$ (Farnworth and Mainville, 2003). Some consumers find kefir too sour to consume on its own and prefer to add flavors and sweeteners, while other consumers prefer to mix frozen fruits with kefir and blend to make a smoothie. Kefir is usually sold with different varieties of fruits and flavors already added (Shah, 2014).

5.3 KOUMISS

Koumiss is a traditional fermented milk product originating in the central Asian steppes and is mostly produced from mare’s milk by spontaneous fermentation of lactose to lactic acid and alcohol (Kosikowski and Mistry, 1997). Koumiss is milky-gray in color and effervescent, with a sharp
alcohol and acidic taste. It contains approximately 2% alcohol, 0.5%–1.5% lactic acid, 2%–4% lactose, and 2% fat (Danova et al., 2005). Although the product is fermented and has a titratable acidity ranging from 0.54% to 1.08%, it is a liquid product showing no curdling. It is widely consumed in western and central Asia (e.g., Mongolia, Kazakhstan, and Kyrgyzstan) and Russia for its nutritive and therapeutic properties. Mare’s milk has important nutritional and therapeutic properties, which are beneficial to the elderly, to convalescents, and to infants (Marconi and Panfili, 1998). The composition of mare’s milk is significantly different from that of bovine milk. It is similar to human milk, in particular regarding its low nitrogen content, its low casein-to-whey protein ratio, and its high content of lactose (Yaygin, 1992; Bonomi et al., 1994). The proteins of mare’s milk are somewhat

<table>
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<tr>
<th>Table 5.1 Chemical Composition of Various Kefirs</th>
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<tr>
<td><strong>Component</strong></td>
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<tr>
<td><strong>CO₂</strong></td>
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<tr>
<td>Polish commercial kefir</td>
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<tr>
<td>Grain fermented, 24 h</td>
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<tr>
<td>Grain free, 24 h</td>
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<tr>
<td><strong>Protein</strong></td>
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<td>Taiwanese kefir from grains (cow’s milk)</td>
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<tr>
<td>Polish lab kefir</td>
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<td><strong>Fat</strong></td>
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<td>Taiwanese kefir from grains (cow’s milk)</td>
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<tr>
<td><strong>Lactose</strong></td>
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<td>Traditional kefir</td>
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<td>Eastern European stir type</td>
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<td>Irish kefir from grains (diluted milk)</td>
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<td>Taiwanese kefir from grains</td>
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<td><strong>Ethanol</strong></td>
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<td>Polish lab kefir</td>
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<td>Irish kefir from grains (diluted milk)</td>
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<td><strong>Lactic Acid</strong></td>
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<td>Traditional kefir</td>
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<td>Eastern European commercial stir type</td>
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<td>Irish kefir from grains (diluted milk)</td>
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different from those of the milk of other species because when the milk is fully renneted, it forms no visible curd, owing to higher whey protein and lower casein content. The fat content of mare’s milk is lower than that of cow’s milk. Traditionally, kumys was made from mare’s milk, but now that mare’s milk is scarce, cow’s milk is used for the preparation. To overcome the difference in the casein and whey protein ratio between mare’s and cow’s milk, a membrane ultrafiltration method is used.

The fermentation of koumiss depends on the action of two distinct types of microorganisms. The major partners in the product are lactic acid bacteria and yeasts (*Kluyveromyces, Saccharomyces, Candida*) (Montanari et al., 1996, 1997). Lactic acid bacteria have been reported to play a major role in fermentation, which affect the aroma, texture, and acidity of the product as well as being of some benefit to human health. Yeast ferments lactose to alcohol, which contributes to the Koumiss product’s typical flavor and taste (Montanari et al., 1996; Viljoen, 2001; Mu et al., 2012). Koumiss harbored yeast populations at 5–7 log cfu/mL.

### 5.3.1 PRODUCTION OF KOUMISS

Like kefir, koumiss is also a slightly alcoholic fermented milk beverage, originally obtained by using a natural mixed starter of lactic acid bacteria and yeasts. However, unlike kefir, koumiss is traditionally prepared from mare’s milk; in addition, in koumiss manufacturing liquid starter is used while in kefir, solid kefir “grains” are used for inoculation. Traditionally, koumiss was manufactured with a part of the beverage of a previous day that was used to inoculate fresh nonpasteurized mare’s milk contained in tanks (“saba” or “turdusk”), which were made from smoked horsehide. Fermentation took place in 3–8 h with the contribution of a mixed indigenous microbial population mainly consisting of *L. delbrueckii* ssp. *bulgaricus*, *L. casei*, *L. lactis* ssp. *lactis*, *Kluyveromyces fragilis*, and *S. unisporus* (Di Cagno et al., 2004; Litopoulou-Tzanetaki and Tzanetakis, 2000; Montanari et al., 1996). Currently, koumiss is manufactured at an industrial level using pure cultures of yeasts and LAB (Tamime et al., 1999).

Considering the increasing popularity of koumiss outside its traditional consumption tract of west and central Asia, coupled with limited availability of horse’s milk, other types of milk including cow’s milk are now used for koumiss preparation. However, mare’s milk contains a low level of casein compared with cow’s milk, and as a result, the fermentate does not coagulate. Therefore, production of koumiss requires some modification of the traditional production practices including adjustment of the chemical composition of cow’s milk or any other to make it comparable to the mare’s milk. Cow’s milk is richer in fat and protein but lower in lactose than mare’s milk. To overcome the difference in the casein and whey protein ratio between horse’s and cow’s milk, various methods have been applied. One approach is to dilute skimmed milk with water to reduce the casein content, and add whey or whey protein concentrate to increase the protein content; also, glucose, sucrose, or lactose hydrolyzed by β-D-galactosidase is added (e.g., to a level of 6.7 g/L) to increase the carbohydrate content of the milk base (Seiler, 2003). Addition of the latter ingredient helps the yeast (*S. cerevisiae*) to grow and become well established in the product (Wszolek et al., 2006). Another approach to adjust the composition and standardization of bovine milk for uniform quality of koumiss production is through the application of membrane technology. By this method, the whey protein content can be adjusted, so that whey protein is concentrated but not lactose. Hence, if cow’s milk is used, the milk is fortified by adding sucrose or modified whey to better approximate the composition of mare’s milk and to allow comparable fermentation. Küçükçetin et al. (2003) reported that a comparable quality of koumiss can be made from bovine
milk modified using membrane technology. The use of ultrafiltration, microfiltration, and nanofiltration to modify the bovine milk and the inoculation of the modified bovine milk with a blend of starter cultures consisting of *L. delbrueckii* ssp. *bulgaricus*, *L. acidophilus*, and *K. lactis* gave a koumiss from bovine milk that was similar to that made from the mare’s milk regarding chemical, physical, and microbiological characteristics.

A general production process of koumiss using bovine milk and mare’s milk is shown in Fig. 5.8. In the commercial manufacture of koumiss from mare’s milk, the bulk starter is prepared first. A yeast culture of

---

**FIGURE 5.8**

Industrial koumiss production from (A) mare’s milk and (B) cow’s milk.

Torula spp. and the LAB culture are inoculated separately into pasteurized skim milk from cow and incubated at 30°C for 15 h and at 37°C for 7 h, respectively. To prepare the bulk starter, the incubated cultures are added to mare’s milk and incubated at 28°C for about 4 days, after which the titratable acidity usually reaches 1.4%. Thirty percent of the bulk starter is added to the fresh mare’s milk at 28°C and the milk is agitated vigorously to introduce air essential for good yeast growth. The fermenting milk is dispensed into bottles with crown-capped closures and incubated for a further 2 h at 20°C.

Large-scale production of koumiss from cow’s milk is carried out by adding 2.5% sugar and 10% starter (L. delbrueckii ssp. bulgaricus, L. acidophilus, and Saccharomyces lactis). The inoculated milk is incubated at 26–28°C until a firm curd forms at the titratable acidity of approximately 0.8%. It is held at approximately 17°C with aeration and intermittent stirring for the optimum yeast growth and, after several hours, the titratable acidity reaches 0.9%. The acidified milk is then bottled. Accumulation of alcohol and carbon dioxide occurs during ripening, and the product is subsequently stored at or below 6°C until sold.

5.3.2 PRODUCT CHARACTERISTICS AND COMPOSITION

Koumiss made from mare’s milk is sweeter than that of cow’s milk. It is milky-green in color, light and fizzy, and has a sharp alcoholic and acidic taste. It possesses a uniform consistency without any tendency to whey off. Lactic acid, ethyl alcohol, and carbon dioxide impart to koumiss the characteristic sourish flavor and fizziness and bubbling on shaking. Because of the high level of whey proteins in mare’s milk, its digestibility is good. Koumiss contains 90% moisture, 2%–2.5% protein (1.2% casein, 0.9% whey proteins), 4.5%–5.5% lactose, 1%–3% fat, and 0.4%–0.7% ash, as well as the main metabolites of fermentation, which are lactic acid (0.7%–1.8%), ethanol (0.6%–2.5%), and CO₂ (0.5%–0.9%). The pyruvic acid, citric acid, acetic acid, and uric acid concentrations of koumiss were reported to be 0.068, 0.91, 0.95, and 0.007 mg/g, respectively (Özer and Kirmaci, 2014). Viable bacterial and yeast count of ~4.97 × 10⁷ and ~1.43 × 10⁷ cfu/mL, respectively, have been reported in koumiss. As in the case of other cultured dairy products, the precise characteristics of koumiss are determined by the starter microorganisms (Özer and Özer, 2000). Comparative detail composition of koumiss from cow’s milk and mare’s milk is given in Table 5.2.

| Table 5.2 Composition of Koumiss Prepared From Modified Bovine Milk, Mare’s Milk |
|-------------------------------------------------|-----------------|-----------------|
| Parameters                                      | Mare’s Milk     | Modified Bovine Milk |
| pH                                              | 6.96            | 6.43             |
| Titratable acidity (mL 0.25N NaOH/100 mL product)| 3.2 ± 0.1       | 6.5 ± 0.1        |
| Total solids (g/100 mL)                         | 9.9 ± 0.1       | 9.1 ± 0.1        |
| Fat (g/100 mL)                                  | 1.0 ± 0.0       | 1.0 ± 0.0        |
| Protein (g/100 mL)                             | 1.6 ± 0.1       | 1.6 ± 0.01       |
| Whey protein (g/100 mL)                        | 0.7 ± 0.1       | 0.71 ± 0.02      |
| Lactose (g/100 mL)                             | 7.3 ± 0.1       | 6.2 ± 0.1        |
| Ash (g/100 mL)                                  | 0.2 ± 0.1       | 0.61 ± 0.02      |
| Density (g/100 mL)                             | 1.033 ± 0.001   | 1.033 ± 0.002    |
| Apparent viscosity (mPas)                       | 2.34 ± 0.20     | 3.12 ± 0.10      |

5.3.3 **CLASSIFICATION OF KOUMISS**

Berlin (1962) and Lozovich (1995) have classified koumiss into three categories based on extent of fermentation (e.g., flavor intensity, level of acidity, and amount of alcohol produced) (Table 5.3). The titratable acidity varies from 1.0% to 1.5% and alcohol content varies from 0.1% to 1.0%. According to Kosikowski and Mistry (1999), the alcohol content varies from 1.0% to 2.5% and lactic acid content varies from 0.7% to 1.8%. Koumiss itself has a low level of alcohol, like small beer, the common drink of medieval Europe. Though koumiss can be strengthened through freeze distillation, it may also be distilled into spirit known as araka or arkhi. Three types of koumiss are produced, viz., strong, moderate, and weak koumiss, depending on the lactic acid content (Dhewa et al., 2015). Strong koumiss is produced by LAB (L. bulgaricus and L. rhamnosus) that acidify the milk to pH 3.3–3.6 and whose conversion ratio of lactose into lactic acid is about 80%–90%. Due to lower pH and high lactic acid content, it falls in the category of strong koumiss. While moderate koumiss contains LAB (L. bulgaricus, L. plantarum, L. casei, and L. fermentum) with restricted acidification properties that lower the final pH to 3.9–4.5 with average sugar conversion ratio of 50%. Light koumiss is a slightly acidified product (pH 4.5–5.0) and is formed by S. thermophilus and Streptococcus cremoris (Dhewa et al., 2015).

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<tbody>
<tr>
<td></td>
<td>Acidity (mL/100 mL)</td>
<td>Alcohol (g/100 mL)</td>
</tr>
<tr>
<td>Weak/sweet-sour/yeasty</td>
<td>0.54–0.72</td>
<td>0.7–1.0</td>
</tr>
<tr>
<td>Medium/boldly sour/yeasty</td>
<td>0.73–0.90</td>
<td>1.1–1.8</td>
</tr>
<tr>
<td>Strong/hardy milk acid/yeasty</td>
<td>0.91–1.08</td>
<td>1.8–2.5</td>
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5.4 **CONCLUDING REMARKS**

Fermentation is the age-old practice of preserving milk by culturing fresh milk and/or leftover milk after daily family consumption, using a small portion of a previous batch/day product as the starter culture. Kefir and koumiss are the nutritious alcoholic beverages produced as a result of yeast-lactic fermentation of milk, originated and traditionally consumed in central Asia and eastern European countries. Because of a number of proclaimed health benefits of these products, their demand is increasing in other parts of the world as well. For the upgradation of manufacturing technology from artisan level to large-scale industrial manufacturing, a great amount of research has been conducted on each and every aspect of product manufacturing technology. However, there are still certain bottlenecks in manufacturing of these products from cow’s milk with traditional taste and texture of conventional products under automated industrial conditions. Considering the increasing demand of these products from other regions, outside the conventional territory, mainly on account of its proclaimed health benefits, it is expected that in the future more concerted research efforts will be made to develop suitable technology to manufacture products from types of other milk (cow and buffalo milk) that would be closely comparable with the conventional products.
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FURTHER READING
CHAPTER 6

REGULATORY ASPECTS OF YOGURT

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6.1 INTRODUCTION

A law is a rule of conduct or action prescribed or formally recognized as binding or enforced by a controlling authority. In the United States, laws are enacted by the US Congress (House of Representatives and the Senate) and signed by the president of the United States of America. The laws dealing with food, drugs and cosmetics are passed by the US Congress and signed by the president (Vetter, 1996).

Regulation is an authoritative rule dealing with details or procedure. It is also a rule or order issued by an executive authority or regulatory agency of a government and having the force of law. For matters dealing with foods, the US Food and Drug Administration (FDA), a part of the US Department of Health and Human Services and the US Department of Agriculture, promulgate rules for administering the law passed by the US Congress. Therefore, regulations are the practical tools by which the laws enacted are enforced. The responsibility of who will enforce the law is stated within the law that is enacted.

The Federal Food, Drug, and Cosmetic Act (FD&C Act) was passed in 1938 and the FDA enforced the laws. The safety of the food is a concern of the producer shipper, processor, and preparer. It must be safe from the standpoint of (1) its physical condition, (2) its chemical content, and (3) the presence of extraneous or foreign materials (i.e., contaminating living microorganisms and/or miscellaneous organic or inorganic debris). Therefore, the safety of the food is closely allied to the condition of the environment in which it is produced, stored, handled, prepared, or served.

The FD&C Act of 1938 does not define “safe” food but rather delineates those things that must not be present in foods that are to be sold, and uses the term food “adulteration” when those conditions are not met. Since the passage of that law, changes in technology and changes in eating and living habits of consumers have added an additional parameter to the safety of food. This concerns the natural chemical content of the foods, which may be greatly altered (or even completely extracted) by processing methods. For example, the milling of grains may totally remove the desirable vitamin content, and the resulting aesthetically appealing bread may, therefore, have little nutritional value unless the extracted vitamins are restored. While such breads would not be unsafe or adulterated in the sense that it would directly cause disease or toxicity, it would certainly be detrimental to the consumer who depended upon it for a complete vitamin source over an extended period of time.

Substances that must be prevented from entering the food include a tremendous variety of chemicals that are used as herbicides, insecticides, fungicides, or fertilizers in the production of plant foods, as well as toxic elements and chemical compounds that may contaminate water supplies utilized for irrigating or cleaning, the harvested foods. The enforcement of regulations preventing such adulteration
has been accomplished by spot testing in certain areas of the country, as well as by the prohibition of the use of certain chemicals in environments where foods are processed or produced.

The first portion of the FD&C Act that one must deal with is the definitions section. This section includes the definition of “foods,” “food additives,” and “labeling” and defines the complete scope of products within the authority of the FDA.

“Food additive” is one of the strangest definitions. It does not mean “food additive” as used and understood by most laypersons or food technologists. What is a “food additive” is commonly understood to mean something added to food, including spices, flavorings, or “foods” such as sugar. The FD&C Act, however, states that a “food additive” is something added to food that is not “generally recognized as safe” or is a substance for which a food additive regulation has been written. Hence, we really have two classes of “additives”: (1) those that are “food additives” under the law and (2) those additives that are or have become generally recognized as safe for intended uses, which thus do not come under the legal definition.

In reality, the definitional section of the food law is not a “definition” section but rather a scope section; it tells us what products the FD&C Act covers. Nonlawyers should be wary of this section because a literal interpretation may not be correct. Section 401 (U.S. FDA, 2015) relating to food standards has an impact on technology by regulating both quality and content of food. Standards may be written to set minimal quantity requirements to specify a minimum quantity of expected ingredients, or to give a specific name to a product.

One purpose of Section 401 (U.S. FDA, 2015) is to protect honest manufacturers who completely fill their containers from those who slack fill. Another purpose is to use food standards to dictate composition. The authority over food products standardized under this provision includes the ability to prescribe a detailed list of every ingredient that can be used in a particular food. Only those products that meet such a standard can be sold under the name of that standardized food. Ice cream is a standardized food; whereas, gelato is not a standardized food. Section 401 was originally used to regulate new food additives on which there was a question of safety. After passage of the 1954 Pesticide Amendment, the 1958 Food Additive Amendment, and the 1960 Color Additive Amendment, all of which established preclearance procedures for these additives, the use of the food standard provision as a means of regulating composition became largely unnecessary.

Section 402 (U.S. FDA, 2015) deals with food adulteration and may be divided between food adulterated because of filth or poisonous and dangerous substances and food adulterated because of “economic inferiority” (Code of Federal Regulations, 2015a,b).

6.1.1 ADULTERATION BY FILTH OR HARMFUL SUBSTANCES, SECTION 402(A) (U.S. FDA, 2015)

The problem of filthy or harmful food was one of the primary reasons for the enactment of the 1906 Pure Food Act. Most of the provisions of the pure food law were reenacted in the 1938 FD&C Act. To a great extent this provision is a basic health law. It prohibits foods from containing either poisonous or deleterious substances or unapproved food additives.

Section 402(a) (U.S. FDA, 2015) also prohibits the sale of foods containing filth or foods held under unsanitary conditions. The latter provision forms the basis for the legal argument of the FDA that it has the authority to define what constitutes unsanitary conditions through a set of good manufacturing practices (GMPs).
6.1.2 ECONOMIC ADULTERATION, SECTION 402(B) (U.S. FDA, 2015)

Economic adulteration relates to economic fraud, the debasement of a genuine article by the addition or substitution of something spurious. Classic examples are the substitution of sugar for fruit in a jam and the substitution of vegetable oil for butter. The FDA has attempted to use this provision to prevent products from being sold that are considered debased or diluted. In most cases, however, the labeling was construed to be “informative” and thus there was no fraud. If Section 402(b) were to be literally applied, no flavor or ingredient could be used that simulated textures or tastes of existing foods. Nevertheless, the law has not been construed in this manner.

6.1.3 FOOD LABELING SECTION, SECTION 403 (U.S. FDA, 2015)

This section contains the “imitation” provision of the law, which together with other labeling provisions, relates to whether or not a product is mislabeled. Attempts have been made to use these labeling provisions separately and collectively to block changes in the nature of the food supply. They have been used, for example, to halt or interfere with the manufacture or sale of products that do not conform to the concept of what is “natural,” i.e., the old established way of making the product. Such a product could make a claim that implied that it was a standardized food but the manufacturer could not utilize the name of the standardized food. A new name would have to be used. The labeling provisions have also been employed in an unsuccessful attempt to make all products that are functionally the same as another established product bear the label “imitation.”

On the whole, most attempts to entirely prohibit the sale of a safe wholesome product have failed. The provisions of Section 403 (U.S. FDA, 2015) are considered by the courts to be strictly labeling laws, specifying how the product must be named. As a general policy, all food products, no matter how composed, may be sold, provided that they are informatively labeled for the consumer.

Many of the provisions of the FD&C Act have been incorporated into the food laws administered by the US Department of Agriculture, including the laws regulating meat, poultry, and egg products.

In addition, the US Environmental Protection Agency now administers all pesticide regulations. The US Consumer Product Safety Commission presently regulates food packaging for hazards other than those related to physiological ingestion. The US Federal Trade Commission (FTC), under its broad mandate to prevent “unfair and deceptive” acts or practices, holds concurrent jurisdiction with the FDA over food labeling. The FTC separately controls food advertising practices. These agencies all work in close concert to regulate food and food packaging.

In a manner similar to the US system of promulgating regulations, the European Union also enacts laws from which regulations are derived. Regulations provide the details of obeying the law. While the details of lawmaking may differ, agencies of the government are responsible for providing details of how to comply with the law.

The objectives of food laws and regulations are to ensure the protection of human health. Thus, regulations provide for the safety of raw materials, storage, processing, packaging, and subsequent operations ending ultimately with the purchase of products by the consumer.

The term “standards” is often used in many parts of the world. Standards in the US context can pertain either to identity or quality. Standards of identity exist for a number of foods. A common or usual name of a food is fixed and specifies ingredients that must be used in the manufacture of the product, their maximum and minimum levels, and compositional requirements of that product. If a food
cannot meet these requirements it cannot be labeled as that food. For example, the word *yogurt* cannot be used if a cultured product does not contain milk.

Standards of quality in the United States are not proscribed. General guidelines have, however, been established to determine if a product is of substandard quality. In other parts of the world, products have to meet certain standards proscribed by regulations. Hence, in these parts of the globe food standard agencies are established and are equivalent to the FDA.

### 6.2 United States Regulations

#### 6.2.1 Food Safety Modernization Act (Public Law 353)

A law passed by the US Congress in 2011 was the first major change in how food is grown, processed, packaged, and distributed in this country. The aims of this legislation are to use science-based methods to improve food production, processing, and consumption and minimize food-borne illness. The regulations to implement this law are being phased in as they also address commodity-specific aspects of the law (*Food and Drug Administration*, 2016).

The major elements of the Food Safety Modernization Act (FSMA) are as follows:

1. Preventive controls
2. Inspection and compliance
3. Imported food safety
4. Response
5. Enhanced partnerships

Preventive controls are mandated across the food supply, and this was new and significant because prior to this legislation, preventive controls were voluntary. Safety plans have to identify and minimize physical, microbiological, and chemical hazards using hazard analysis critical control plan methodology. The details include not only identifying critical control points but also methodology to verify that the controls are working and keeping records of testing and results. These preventive controls will vary by the type of food in question so that the regulations will be specific for the type of food. These controls will also be implemented at the farm level where food originates. Food companies are held accountable for preventing contamination, i.e., companies manufacturing contaminated food will be sued in a court of law, holding the chief executive officers responsible. Monitoring identified points are important as are written procedures for corrective actions and records. Furthermore, preventive controls should be continuously monitored for their effectiveness.

The inspection and compliance part of FSMA recognizes that inspection is an important tool in producing safe food. The FDA will use a risk-based approach in its inspections by innovating to achieve the most thorough, efficient, and effective means with existing resources. Imported foods will be more thoroughly scrutinized by the FDA. Regulations will require importers to verify suppliers’ activities to ensure that the food is safe. The FDA can refuse admission of imported food if the foreign country refuses to allow FDA inspections of foreign facilities. Until this legislation was enacted, food recalls were voluntary. Now FSMA gives the FDA mandatory recall authority. It is expected that the FDA will invoke this authority infrequently because most food manufacturers are largely compliant. This provision of FSMA, however, provides a critical tool for the FDA to protect public health.
Supply chain programs are provided greater flexibility in meeting compliance with FSMA. The rule mandates that a manufacturing/processing facility have a risk-based supply chain monitoring program for those raw materials identified as a hazard. Food manufacturing facilities are responsible for ensuring that raw materials and ingredients are received only from approved suppliers. Suppliers and brokers/distributors can conduct supplier verification activities. The receiving facility must review and assess that entity’s documentation and control of hazards. Current good manufacturing practices (CGMPs) are updated and clarified. Some of the previous nonbinding provisions, e.g., education and training of employees, are now mandatory. It is management’s duty to ensure that employees involved in manufacturing, processing, packaging, and holding food are qualified to perform their assigned duties. The FDA’s long standing position on CGMPs addressing allergen cross contamination is now explicit in the text of the regulations. Compliance dates for businesses are staged over a number of years. Very small businesses (with sales of less than $1 million) have 3 years to implement these changes, but they must keep records to prove that they meet the requirements of a very small business immediately. Small businesses (fewer than 500 full-time equivalent employees) have 2 years to comply with the new regulations. All other businesses, except those subject to the Pasteurized Milk Ordinance (PMO), have 1 year to comply with the new regulations. Businesses subject to the PMO have 3 years to meet the new requirements.

6.2.2 DEFINITION OF MILK, GRADE A MILK, AND CULTURED MILK (CODE OF FEDERAL REGULATIONS, 2015A,B)

When the word milk is used in the United States, it is cow’s milk. Milk from other mammalian species is preceded by the name of the species, e.g., buffalo’s milk, goat’s milk, etc. Milk means the lacteal secretion, practically free from colostrum, obtained by the complete milking of one or more healthy cows, which may be clarified and may be adjusted by separating part of the fat therefrom: concentrated milk, reconstituted milk, and dry whole milk.

1. Nonfat milk means skim milk, concentrated skim milk, reconstituted skim milk, and nonfat dry milk. Water, in a sufficient quantity to reconstitute concentrated and dry forms, may be added.

2. Cream means cream, reconstituted cream, dry cream, and plastic cream. Water, in a sufficient quantity to reconstitute concentrated and dry forms, may be added.

This embodies the welfare of the animals. In order to ensure a safe, readily available supply of milk and milk products, milk producers, processors, and government/regulatory agencies are involved.

The United States Public Health Service, a division of the FDA, develops and maintains the Standard Milk Ordinance. This is a voluntary program for adoption by state and local health and agricultural departments. This ordinance provides model ways to milk producers for housing, feeding, milking, and on-farm storage of milk. The Standard Milk Ordinance and its companion ordinance called the Grade “A” PMO were combined and are simply called Grade “A” PMO (USDHHS, 2015). These specifications are incorporated in the procurement of milk and milk products shipped across state lines (interstate). Since milk from several farms is collected in the same tanker transport, samples from individual farms are tested using methods described in the Standard Methods for the Examination of Milk and Milk Products published by the American Public Health Association. All milk, whether Grade A or otherwise, should contain less that the limits set by FDA for pesticides, herbicides, and drug residues.
According to the PMO, the appearance and odor of milk shall not show any abnormal conditions (e.g., clotting, ropy, bloody, mastitis) and the odor shall be free from any objectionable feed or other off odors. The somatic cell count shall be below 750,000 per mL, shall be free from β-lactam or other antibiotic residues, have a sediment level not exceeding 1.5 mg, and a bacterial count of less than $5 \times 10^5$/mL. Processors receiving raw milk may establish their own standards that are more stringent than the ones proscribed. The milk is pumped from the tankers only after these tests are satisfactory. The milk is transferred to a raw milk silo.

All food products have to be processed in an approved facility and according to CGMPs. For a factory producing cultured dairy foods, the design has to provide separate rooms for operations. In one room, pasteurizing, processing, and cooling take place, and there is a separate room for packaging, another for storage and cleaning of packaging containers, and yet another for cleaning and sanitizing equipment. There are also strict rules for approved processing equipment, containers used in the processing rooms, pipe joints, mix-proof valves, tanks, and their cleanability and sanitizability. All these details are provided in the PMO.

When the word *pasteurized* is used to describe a dairy ingredient, this means that every particle of such ingredient shall be heated in properly operated equipment to one of the temperatures specified and held continuously at that temperature or higher for a specified time. The times and temperatures are listed in Table 6.1.

All processing steps must ensure that at no time raw milk comes in direct contact with pasteurized milk. This is achieved by maintaining a pressure differential between the pasteurized and raw side of at least 2 pounds per square inch (psi) or 13.79 kilo Pascal (kPa). Pressure is developed when the milk passes through a timing pump. A homogenizer can serve as a timing pump, as can a positive displacement pump. According to the PMO, the flow rates and temperatures reached in the pasteurization section of a high temperature short time (HTST) is checked by state officials every 3 months and they place a seal on the timing pump. This seal, if broken in an emergency by the HTST operator, requires retesting and resealing of the system by the state inspector. The holding tube should be at an incline, and at the end of the holding tube, a calibrated mercury thermometer is positioned followed by a dual-flow diversion valve and a pressure differential recorder. The chart where the temperature history of the product being processed is recorded is called the thermal safety limit recorder. The tracings have to be retained for 90 days, and each chart must contain the date, name of the operator, and number of the

<table>
<thead>
<tr>
<th>Temperature</th>
<th>°F</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
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</tr>
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<td>15 s</td>
</tr>
<tr>
<td>89</td>
<td>191</td>
<td>1.0 s</td>
</tr>
<tr>
<td>90</td>
<td>194</td>
<td>0.5 s</td>
</tr>
<tr>
<td>94</td>
<td>201</td>
<td>0.1 s</td>
</tr>
<tr>
<td>96</td>
<td>204</td>
<td>0.05 s</td>
</tr>
<tr>
<td>100</td>
<td>212</td>
<td>0.01 s</td>
</tr>
</tbody>
</table>
processing plant. The time and temperature requirements for pasteurization for various products are listed in the PMO (Table 6.1). Time and temperature are increased for high solids, high fat, and viscous mixes.

Since pasteurization equipment can be batch or continuous, only continuous HTST equipment is being considered in this chapter. Further, continuous processes can be configured in many different ways. As an example, two such configurations are illustrated in Figs. 6.1 and 6.2. The first schematic uses newer technology by incorporating an electromagnetic flow meter as the timing device, often abbreviated as “magflow meter.” Magflow meters have no moving parts and operate on the principle of Faraday’s law. The second schematic illustrates the use of a positive displacement pump as the timing device. There are a number of ways HTST systems are configured, but the principles outlined here are applicable to all systems. The enforcement of these regulations has been delegated to individual state inspectors. In some states the pasteurizer operator has to be certified, and certification is obtained by attending a course and passing an exam dealing with pasteurization.

*Cultured milk* is another term used in regulations (U.S. FDA, 2015, 21 CFR § 131.112). Cultured milk is defined as a product obtained by adding characterizing microbial organisms to milk, partially skimmed milk, skim milk, or cream. These fluid products must be pasteurized or ultrapasteurized prior to adding cultures and may be homogenized. Cultured milk may contain other optional ingredients. Optional ingredients of dairy origin are concentrated skim milk (also called skim concentrate), nonfat dry milk (skim milk powder), buttermilk, whey, lactose, lactalbumin, lactoglobulin, and modified whey.

**FIGURE 6.1**
Configuration of a high-temperature short-time (HTST) system using a magnetic flow meter as a timing device with an in-line homogenizer prior to the product entering the heating section.
(whey protein concentrate, whey protein isolate, partially delactosed whey, partially demineralized whey, and partially delactosed and partially demineralized whey or their powders). Even though a reading of the Code of Federal Regulations provides limits to the amounts of optional ingredients, it was stayed by a court. Nondairy optional ingredients include acidifying agents, nutritive carbohydrate sweeteners, flavorings, colorings, stabilizers, aroma-producing cultures, salt, and flavor precursors, e.g., citric acid.

Cultured milk must contain a minimum of 3.25% milkfat, 8.25% milk solids-not-fat, and a titratable acidity of 0.5% expressed as lactic acid. Cultured milk can be fortified with vitamins A and D. If the milk was homogenized the label may indicate “homogenized.”

6.2.3 YOGURT REGULATIONS (U.S. FDA, 2015; 21 CFR § 131.160)

Yogurt is a dairy product produced by culturing with lactic acid bacteria, Lactobacillus delbrueckii ssp. bulgaricus and Streptococcus thermophilus, and one or more of the following: cream, milk, partially skimmed milk, skim milk, and reconstituted dairy ingredients. Other lactic acid–producing bacteria may also be included along with the two primary bacteria just named. The yogurt mix has to be pasteurized and may be homogenized prior to the addition of cultures. Flavoring ingredients may also be added.
postpasteurization. The product may be heat-treated post fermentation thus extending the shelf life. Even though the regulation says that the milkfat level has to be a minimum of 3.25%, this provision has been stayed by the courts. Yogurt must contain a minimum of 8.25% milk solids-not-fat and may be fortified with vitamins A and D. The regulation as printed also states that there must be a minimum titratable acidity 0.9% measured as lactic acid. This acidity level has also been stayed by the courts pending outcome of public hearings. Yogurt regulations do allow for optional ingredients of both dairy and nondairy origins. Dairy ingredients must be derived from Grade A milk. Among the optional dairy ingredients are concentrated skim milk, nonfat dry milk, buttermilk, whey, lactose, lactalbumin, lactoglobulin, whey, partially demineralized whey, partially delactosed whey, partially delactosed and demineralized whey, whey protein concentrate, whey protein isolate, and lactose. Nondairy ingredients permitted in yogurt include nutritive carbohydrate sweeteners, malt extract, honey, maple sugar, dextrose, glucose syrup, sorghum syrup, flavoring ingredients, color additives, stabilizers, and preservatives as functional ingredients. The name of the product shall be called yogurt (acceptable alternate spelling “yogourt” and “yoghurt”). If the product is heat-treated after culturing, the name of the food shall be followed by the parenthetical phrase “(heat-treated after culturing).” If vitamins are added, the phrase as appropriate should be used: “vitamin A” or “vitamin A added.” Similarly, if vitamin D is used, “vitamin D” or “vitamin D added” is used. If both vitamin A and D are involved, “vitamin A and D” or “vitamin A and D added” is used. If flavoring is used, the common and usual name of the product should indicate this, e.g., vanilla yogurt or strawberry yogurt.

When the word milk is used in consumer products it is meant be milk standardized to a minimum milkfat content of 3.25% and 8.25%. Similarly, if the word yogurt is used, it represents milk being fermented by L. delbrueckii ssp. bulgaricus and S. thermophilus. Low-fat yogurt is also a standardized food and meets all the requirements for yogurt except the milkfat content can be from 0.5% to 2.0% prior to the addition of bulky flavors. The milkfat content must be declared in nondecimal fractions, e.g., ½% instead of 0.5%. Nonfat yogurt is also a standardized food that meets all the requirements for yogurt except the fat content must be less than 0.5% prior to the addition of bulky flavors.

Since the stayed provisions of yogurt regulations can be confusing, they can be summarized as follows:

1. There are no restrictions of milk-based ingredients that can be added to boost the milk solids-not-fat above a minimum of 8.25%.
2. Reconstituted dairy ingredients can be used as basic ingredients in yogurt manufacture.
3. Preservatives can be added to yogurt.
4. There is no minimum titratable acidity.
5. The minimum fat requirement of 3.25% is not necessary if the milk solids-not-fat level is boosted above 8.25% by the addition of optional ingredients.

Debates over live and active cultures, minimum numbers of yogurt organisms per unit, or changing standards to include minimum pH value in addition to titratable acidity have not yet been made a part of the yogurt regulations. All standards for yogurt apply to yogurt drinks as well.

6.2.4 LABELING

To protect and inform consumers, the FDA requires truthful food labeling (U.S. FDA, 2013a,b). Because federal laws preempt state regulations, labeling is uniform throughout the nation. Any product
that crosses state lines for the purposes of sale (interstate commerce) comes under federal laws. Only products manufactured (including all ingredients and packaging) and sold within a state are exempt from federal laws. Some state laws are not preempted by federal regulations, including warning labels, open date coding, unit price labeling, food grading, recycling container deposit labeling, and item price labeling. States can also continue to establish and enforce standards of identity for products that do not have federal standards of identity, e.g., frozen yogurt or yogurt smoothies.

There are a number of aspects of types of labeling to include product nomenclature, flavor labeling, ingredient declaration, allergen labeling, nutrition facts panel, and special labeling requirements. Food labeling regulations specify what information must appear on the package label, where it must be placed, the format of the label, and the size of mandatory label material, e.g., nutrition facts panel. Information has to be provided on the surface that the consumer is most likely to see during retail display. This part of the package is called the principal display panel (PDP). The PDP must show the product name and flavoring, and the volume or net weight of the product in the container. On the right of the PDP is the information panel (IP). The IP must show a complete ingredient listing in decreasing order of preponderance; name and place of business of the manufacturer, packer, or distributor; the nutritional facts panel; and, if appropriate, specific requirements related to nutrient content claims, warnings, or statements of special dietary use. For dairy products following Grade A requirements, all packages must be conspicuously marked “Grade A” and show the identity of the plant where milk was processed, condensed, and/or dried, either reconstituted or recombined.

6.2.4.1 Product Nomenclature
The name of the food or statement of identity may be regulated via the standard of identity specified in federal regulations, or if no federal standard exists, by the state’s standard of identity. If neither federal nor state standards of identity exist for the food, then the common and usual name readily understood by the consumer must be used. Cultured milk and yogurt have federal standards of identity and therefore cannot use descriptive names. Descriptive names must be suggestive enough to reveal the basic composition of the product that avoids any confusion with what the product really is. The descriptive name must be understood by the consumer. If a product is called a yogurt smoothie, it must contain a descriptor like a blend of yogurt and fruit juice/ juices.

6.2.4.2 Flavor Labeling
There are six different aspects to the labeling of flavors. Three of the six aspects are concerned with flavors that are added in a liquid form, also called “from the bottle.” The first aspect deals with flavors that are extracted from botanic sources that have the primary characterizing flavor as a part of this extraction and is all natural. For example, yogurt to which vanilla extract is added as a flavoring is labeled vanilla yogurt. The second aspect deals with flavoring that is added and contains two or more natural flavors. In addition to the principal characterizing flavor, another natural flavor is also present to reinforce the principal characterizing flavor. Yogurt flavored with coffee extract in which other natural flavors reinforce the coffee flavoring should be labeled as “coffee yogurt with other natural flavors.” The third aspect deals with instances where the natural flavors used are not extracted from sources determined to be a natural source of the characterizing flavor or an artificial flavoring simulates, resembles, or reinforces the declared characterizing flavor. Vanilla flavor to which vanillin has been added in a proportion that the vanillin is greater than the impact vanilla extract would come under this aspect. Yogurt flavored with this type of flavor has to be labeled as “artificially flavored vanilla yogurt.”
The next two aspects of flavor labeling deal with instances where consumers expect to have characterizing food ingredients (fruits, for instance). The characterizing fruit may not be present in sufficient amounts to flavor the product. In the fourth instance, yogurt that contains some fruit but not in an amount sufficient to impart its characterizing flavor and is fortified with a natural extract of the same fruit should be labeled as “peach-flavored yogurt.” The word flavored is preceded by the name of the fruit that was added. The fifth aspect deals with instances where the food contains a food ingredient in quantities not sufficient to impart enough characterizing flavor and the flavor has to be boosted by the addition of natural flavors that simulate or reinforce the characterizing flavor. Yogurt containing such a flavor system should be labeled “peach yogurt with other natural flavors” to reflect that a part of the flavor comes from the addition of peaches and also contains other natural flavorings that help with the flavor intensity of such a yogurt.

The last aspect deals with foods that have sufficient amounts of characterizing food ingredient to flavor the finished product, for example, yogurt that contains enough strawberries to impart its flavor. Such a product will be labeled as “strawberry yogurt.” No other natural or artificial flavors are added to this yogurt to impart the flavor except the strawberries.

6.2.4.3 Ingredient Declaration

Food products that contain more than one ingredient sold in retail must have an ingredient declaration. The ingredient must be declared by its common name understood by the consumer. There are specific regulations for colors, sweeteners and incidental additives, processing aids, and lipid ingredients (fats and oils). All certified colors must be included by name, and any beverage product containing fruit or fruit juices must declare the percentage of fruit or fruit juice in that product.

Products with federal standards of identity allow for the grouping of ingredients or must provide a common or usual name for the ingredient. For example, skim milk, concentrated skim milk, and nonfat dry milk can all be labeled as “skim milk or nonfat milk.” Similar groupings allow for milk, buttermilk, cream, whey, and butteroil/anhydrous milkfat. The ingredient statement must appear prominently on the IP or the PDP. The entire list must appear without any breaks for noningredient copy. The size of the font has to be at least 1/16 of an inch (1.59 mm). Ingredients may be listed in groupings or in a dispersed manner. It is thought that consumers may find grouping of ingredients easier to understand. For example, strawberry yogurt with granola topping can be labeled using the grouping method as: yogurt (cultured milk, strawberries, sugar, gelatin, pectin, and natural flavors) and granola (oats, puffed rice, corn syrup, brown sugar, raisins, and almonds). The ingredient label for this same yogurt using the dispersion method may read: cultured milk, sugar, oats, corn syrup, strawberries, puffed rice, brown sugar, raisins, almonds, gelatin, pectin, and natural flavors. In this method, all ingredients are listed by their common and usual name in decreasing order by weight, and the multicomponent food itself is not listed.

6.2.4.4 Allergen Labeling

In 2004 the US Congress passed the Food Allergen Labeling and Consumer Protection Act. Starting January 1, 2006, all food products containing allergens had to be labeled as to the type of allergen present in the food. The eight most commonly recognized allergens were milk, eggs, fish, shellfish (crustaceans), tree nuts (almonds, walnuts, pecans, etc.), peanuts, wheat, and soybeans. The allergen labeling law is applicable to both domestic and imported foods. The act was prompted by a 2013 estimate by the Centers for Disease Control of 30,000 annual emergency room visits, 2000 hospitalizations, and 150
deaths in the US alone. The law requires that food labels identify the food source names of all major food allergens used in the formulation of the food. This requirement is met if the common or usual name of an ingredient that is a major food allergen already identifies the allergen’s food source name. For example, if yogurt was labeled using the dispersion method, it would indicate cultured milk. In this instance milk is one of the eight major allergens identified in the term “cultured milk.” Allergens can be identified in one of two ways: in parentheses after the name of the ingredient, e.g., lecithin (soy) or whey (milk); or alternately, the allergen is listed after or next to the ingredient label in a “contains” statement, for example, “contains soy and milk.” Consumers may suffer from allergies other than the identified eight major allergens. It is advisable for consumers to read ingredient labels with care to avoid the food allergen in question. In manufacturing facilities, more than one allergen may be processed on the same line leading to a possibility of cross contamination. The FDA advises food manufacturers to use advisory statements such as “may contain [allergen]” or “produced in a facility that also uses [allergen].” Such labels are not a substitute for GMPs.

6.2.4.5 Nutritional Labeling

In 1990, the US Congress enacted the Nutritional Labeling and Education Act (NLEA), which made it mandatory for all packaged food sold in retail commerce to clearly show the nutritional content of a serving of the food on a specified formatted nutritional facts panel. The serving size is the normal customary amount of that food consumed per eating occasion by a person 4 years of age or greater. The serving size should be expressed in common household units as appropriate for the food. For liquids, the serving size may be expressed in volumetric terms as cups or fractions thereof in fluid ounces (fl oz). For liquids, a cup represents 8 fl oz or approximately 240 mL. For solid foods, the common household measure is expressed in cups or fraction thereof as weight ounces, and the weight is dependent on the density of the food in question. The FDA established reference amounts of food consumed for 100 food product categories. The established reference amount consumed is the benchmark for the serving size declared on the label and expressed in common household measures (cups, tablespoons, teaspoons). The serving size is immediately followed by parentheses containing the metric equivalent of the serving (mL or g). Barring exceptions, the nutrient amounts must express the amounts of nutrients in one serving of the food. The total number of servings per packaging unit has to be indicated below the serving size. The nutrient content is presented in the context of a total daily diet. The accepted American diet consists of a daily consumption of 2000 calories (8368 kJ). From this theoretical diet, recommended intake levels or daily values (DVs) of individual nutrients have been developed based on current dietary guidelines. These values are reported as quantity per serving (g or mg) and also as a percentage of the DV for the nutrient. The nutritional facts box must contain each of these elements: total calories (in bold type), total fat, saturated fat, trans fat, cholesterol, sodium, total carbohydrates subcategorized as dietary fiber, total sugars, added sugars, protein, vitamin D, calcium, iron, and potassium. As announced by the FDA on May 27, 2016, the reference amount customarily consumed for yogurt is 1 cup or 225 g. The new nutritional facts panel is different from the old format (Table 6.2). The new panel has a bolder and larger type for servings. The serving sizes are updated and the calorie content per serving is in a larger and bolder type. The daily values are updated, and under total carbohydrates, in addition to fiber and total sugars, the amount of added sugar has to be indicated. There is a change in the micronutrient type, and the actual amounts have to be declared. The new footnote required states, “The % Daily Value (DV) tells you how much a nutrient in a serving of food contributes to a daily diet. Two thousand (2000) calories a day is used for general nutrition advice.”
Package size affects what people eat. So packages containing between one and two servings will be required to be labeled as containing one serving, e.g., a 15-oz can of soup is usually consumed at one sitting and therefore is considered as one serving, and the nutrients in that container must be declared. For certain products that are larger than one serving but that could be consumed in one sitting or multiple sittings, manufacturers must provide dual column labeling indicating the amount of calories both on a per serving basis and per package or per unit basis (Tables 6.4 and 6.5).

### 6.2.4.6 Other Regulations Governing Voluntary Labels

There are additional symbols and logos that yogurt manufacturers may opt to use. These include the “Real” seal instituted by the Dairy Management Inc. to distinguish between authentic dairy products vis-à-vis simulated dairy products. “Live and Active” is another symbol that can be licensed from the...
Table 6.3 Daily Reference Values Revised in 2015

<table>
<thead>
<tr>
<th>Component</th>
<th>Daily Reference Value</th>
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<tbody>
<tr>
<td>Total fat</td>
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<tr>
<td>Saturated fats</td>
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</tr>
<tr>
<td>Trans fats</td>
<td>0 g</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>300 mg</td>
</tr>
<tr>
<td>Sodium</td>
<td>2400 mg</td>
</tr>
<tr>
<td>Total carbohydrate</td>
<td>300 g</td>
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<tr>
<td>Dietary fiber</td>
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<tr>
<td>Total sugars</td>
<td>Not specified</td>
</tr>
<tr>
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<tr>
<td>Protein</td>
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<tr>
<td>Vitamin D</td>
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</tr>
<tr>
<td>Calcium</td>
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<td>Iron</td>
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</tr>
<tr>
<td>Potassium</td>
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</tr>
</tbody>
</table>

Table 6.4 Dual Column Nutritional Facts Panel

**Nutrition Facts**

2 servings per container

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<thead>
<tr>
<th>Serving size</th>
<th>Per serving</th>
<th>Per container</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>220</td>
<td>440</td>
</tr>
<tr>
<td>Calories</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total Fat</td>
<td>5g</td>
<td>10g</td>
</tr>
<tr>
<td>Saturated Fat</td>
<td>2g</td>
<td>4g</td>
</tr>
<tr>
<td>Trans Fat</td>
<td>0g</td>
<td>0g</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>15mg</td>
<td>30mg</td>
</tr>
<tr>
<td>Sodium</td>
<td>240mg</td>
<td>480mg</td>
</tr>
<tr>
<td>Total Carb.</td>
<td>35g</td>
<td>70g</td>
</tr>
<tr>
<td>Dietary Fiber</td>
<td>6g</td>
<td>12g</td>
</tr>
<tr>
<td>Total Sugars</td>
<td>7g</td>
<td>14g</td>
</tr>
<tr>
<td>Incl. Added Sugars</td>
<td>4g</td>
<td>8g</td>
</tr>
<tr>
<td>Protein</td>
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<td>18g</td>
</tr>
<tr>
<td>Vitamin D</td>
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<td>10mcg</td>
</tr>
<tr>
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<td>400mg</td>
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<td>Potassium</td>
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<td>940mg</td>
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</table>

* The % Daily Value (DV) tells you how much a nutrient in a serving of food contributes to a daily diet. 2,000 calories a day is used for general nutrition advice.
National Yogurt Association, which stipulates that at the time of manufacture the product contained a minimum of 10 million culture organisms per gram of the product. Then there are dietary law symbols such as “Kosher” and “Halal” to certify that the product meets religious dietary guidelines. The religious certification involves inspection of facilities, ingredients, processing, and packaging with regard to their codes for dietary practices. All products sold in retail must carry a universal product code (UPC) for use by laser scanners. Data coded in the UPC has three main goals: company prefix uniquely assigned to a company, an item reference number uniquely created for a product, and a check digit to check the accuracy of the information in the barcode when the item is scanned. Lastly, there is code dating. This is not required by the FDA but it is required by many states. Code dating is also referred to as “sell by date.” The code printed on a product container is used to track and identify the date of production, location of the plant where it was produced, and filling line or processing line. This is helpful in inventory control, rotation of products, and in product retrieval in case of a recall (U.S. FDA, 2013a,b).

### 6.3 CODEX ALIMENTARIUS STANDARDS

The United Nations Economic Commission for Europe (UNECE) established the Geneva Protocol in which a harmonized layout for food commodity standards was proposed. The relevant working party in UNECE provides quality standards for fresh fruit and vegetables and other food

<table>
<thead>
<tr>
<th>Component</th>
<th>Claim</th>
<th>Condition (Not More Than)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Energy</td>
<td>Low</td>
<td>40 kcal (170 kJ) per 100 g (solids) or 20 kcal (80 kJ) per 100 mL (liquids)</td>
</tr>
<tr>
<td></td>
<td>Free</td>
<td>4 kcal per 100 mL (liquid)</td>
</tr>
<tr>
<td>Fat</td>
<td>Low</td>
<td>3 g per 100 g (solids) or 0.75 g per 100 mL (liquid)</td>
</tr>
<tr>
<td></td>
<td>Free</td>
<td>0.5 g per 100 g (solid) or 0.01 g per 100 mL (liquid)</td>
</tr>
<tr>
<td>Saturated fat</td>
<td>Low</td>
<td>1.5 g per 100 g (solid), 0.75 g per 100 mL (liquid) and 10% of the energy</td>
</tr>
<tr>
<td></td>
<td>Free</td>
<td>0.1 g per 100 g (solid), 0.1 g per 100 mL (liquid)</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>Low</td>
<td>0.02 g per 100 g (solid), 0.01 g per 100 mL (liquid)</td>
</tr>
<tr>
<td></td>
<td>Free</td>
<td>0.005 g per 100 g (solid), 0.005 g per 100 mL (liquid), and for both claims less than 1.5 g saturated fat per 100 g (solid), 0.75 g per 100 mL (liquid), and 10% of energy of saturated fat</td>
</tr>
<tr>
<td>Sugar</td>
<td>Free</td>
<td>0.5 g per 100 g (solid), 0.5 g per 100 mL (liquid)</td>
</tr>
<tr>
<td>Sodium</td>
<td>Low</td>
<td>0.12 g per 100 g</td>
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<tr>
<td></td>
<td>Very low</td>
<td>0.04 g per 100 g</td>
</tr>
<tr>
<td></td>
<td>Free</td>
<td>0.005 g per 100 g</td>
</tr>
<tr>
<td>Protein</td>
<td>Source</td>
<td>10% nutrient reference value (NRV) per 100 g (solid), 5% NRV per 100 mL (liquid) or 5% of NRV per 100 kcal (12% NRV per MJ) or 10% NRV per serving</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>2×the values for source</td>
</tr>
<tr>
<td>Vitamins and minerals</td>
<td>Source</td>
<td>15% NRV per 100 g (solid), 7.5% NRV per 100 mL (liquid) or 5% NRV per 100 kcal (12% NRV per MJ) or 10% NRV per serving</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>2×the source value</td>
</tr>
</tbody>
</table>
commodities moving in trade in Europe, with the objective of preventing disputes over the handling of these products during transport. The layout still forms the basis of most food commodity standards worldwide including Codex standards. Cooperation between the Food and Agriculture Organization (FAO) of the United Nations, Codex, and UNECE went through different stages (e.g., FAO/UNECE work on fruit juices, later Joint Codex/UNECE meetings on fruit juices and quick frozen fruits and vegetables, which were later taken over by Codex). Close cooperation between the two bodies continues until today on standards for fresh fruits and vegetables. After the International Dairy Federation had initially worked on standards and labeling requirements for milk and milk products, the work was taken up by the Joint FAO/World Health Organization (WHO) Committee of Government Experts on the Code of Principles Concerning Milk and Milk Products. The committee developed formal procedures for the elaboration of its standards, which involved consultation with governments between meetings of the committee itself and which are still used today by the Codex Alimentarius Commission. Regional efforts to harmonize national food standards had begun after World War II. In Latin America, Carlos Grau of Argentina promoted the idea of a Código Latino-Americano de Alimentos. The idea of a Europe-wide Codex Alimentarius based on the Codex Alimentarius Austriacus was actively pursued by Hans Frenzel of Austria between 1954 and 1958. Frenzel’s work culminated in the creation of the Council of the Codex Alimentarius Europaeus in June 1958 under the joint sponsorship of the International Commission on Agricultural Industries and the International Bureau of Analytical Chemistry. Progress made by the council was not rapid, and in August 1960, the council proposed to WHO that it should associate itself with that organization, and WHO referred the matter to the FAO for discussion of the outlines of how an agreement to take over the work could be reached. Currently the Codex Alimentarius Commission has promulgated standards for many foods, including fermented milk, as the commission aims to harmonize food trade around the globe.

Codex Alimentarius has clearly defined standards for milk production, collection from farms, delivery to the dairy plant, storage, and pasteurization. It also has standards for cleaning and sanitation in dairy plants (Anon, 2011).

### 6.3.1 Definitions of Fermented Milk

Fermented milk is a product obtained by fermenting milk with or without compositional modification by the action of suitable microorganisms (Codex Standard, 2003). This definition applies to reconstituted and recombined milk that is subject to fermentation by suitable microorganisms. The fermentation may or may not coagulate the milk. The added cultures shall be viable, active, and abundant in the product to the end of indicated shelf life date shown on the package. For products that are heat-treated postfermentation, these requirements do not apply. Some types of fermented milk are characterized by specific culture organisms used for fermentation; yogurt must be fermented by the symbiotic relationship of *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus*. Yogurt can also be fermented by *S. thermophilus* in combination with any *Lactobacillus* species. Acidophilus milk shall be fermented by adding a culture of *Lactobacillus acidophilus*. Kefir shall be fermented by a starter culture prepared from kefir grains, *Lactobacillus kefiri*, species of the genera *Leuconostoc*, *Lactococcus*, and *Acetobacter* grow in a strong specific relationship. Kefir grains are made up of lactose fermenting yeasts, *Kluyveromyces marxianus*, and nonlactose fermenting yeasts, *Saccharomyces unisporus*,...
Saccharomyces cerevisiae, and Saccharomyces exiguus. Kumys or kumis culture contains L. delbrueckii ssp. bulgaricus and K. marxianus.

Codex defines concentrated fermented milk as a milk product with an increased protein content prior to or after fermentation to a minimum protein content of 5.6%. Traditional concentrated fermented milk product includes strained yogurt (also called Greek-style yogurt in the United States), labneh, ymer, and ylette. Flavored fermented milk products are composite products containing a maximum of 50% of nondairy ingredients (sweeteners, fruits, vegetables, etc.). Drinks based on fermented milk are also composite products obtained by mixing fermented milk with potable water with or without the addition of other ingredients containing a minimum of 40% fermented milk.

### 6.3.2 CODEX STANDARDS FOR YOGURT (YOUGURT)

Codex Standard A-11(a) (1975) defines yogurt as a coagulated milk product obtained by the lactic fermentation through the action of *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus*. The microorganisms in the final product must be abundant and viable. Sweetened yogurt is yogurt to which one or more sugars have been added. Sugars are defined in the Codex as any carbohydrate sweetening matter. A product labeled “yogurt” or “yoghurt” must contain a minimum of 3% fat and 8.2% milk solids-not-fat. Partially skimmed yogurt must have a fat content between 0.5% and 3% and a milk solids-not-fat minimum of 8.2%. Skimmed yogurt must contain less than 0.5% and minimum milk solids-not-fat content of 8.2%. For sweetened yogurt, partially skimmed or skimmed yogurt the minimum composition must be met for the milk part of the product. The yogurt culture must contain *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus*. The standards permit optional ingredients to include milk powder, skimmed milk powder (nonfat dry milk), buttermilk, concentrated whey, whey powder, whey proteins, whey protein concentrates, water-soluble milk proteins, casein, and caseinates all manufactured from pasteurized products. Other suitable cultures may be added in addition to the two specified. Sugars are permitted in sweetened yogurt.

A complete list of ingredients must be declared on the label in decreasing order of preponderance. The net contents are declared by weight either using the metric system or avoirdupois or both systems of measurement. Volumetric net content declaration using either or both systems is also permitted as required by the country in which the product is sold. The name and address of the packer, distributor, importer, exporter, or vendor shall be declared. The country of manufacture of the food shall be declared except that foods sold within the country of manufacture need not declare the country of manufacture. There shall be a clear marking of the date of production, i.e., the date the final product was packaged for sale or the sell-by date or “minimum durability date.” Each container shall be permanently marked in code or clearly to identify the producing factory and the lot.

Food additives are also permitted in fermented milk in accordance with General Standards for Food Additives (Codex Standard 192-1995). Additional additives may be presented in fermented milk products as a carryover from nondairy ingredients. The approved additives are classified as acid regulators, carbonating agents, colors, emulsifiers, flavor enhancers, packaging gases, preservatives, stabilizers, sweeteners, and thickeners. Unflavored yogurt cannot contain emulsifiers, flavor enhancers, acidity regulators, colors, packaging gases, preservatives, sweeteners, stabilizers, thickeners, or carbonating agents.

The European Union and many other countries have adopted the Codex Alimentarius Standards.
6.4 HEALTH CLAIMS

Health claims describe a relationship between a food or food component and the reduced risk of diseases or health-related conditions. It is a general belief that “we are what we eat” and that some foods are helpful in certain health conditions. Various herbs and dietary supplements are marketed for these properties with or without scientific validation of the properties of these compounds. Thus, a careful line should be drawn between foods and drugs. A drug is defined as a substance recognized by an official pharmacopoeia or formulary; a substance intended for use in the diagnosis, cure, mitigation, treatment, or prevention of disease; and a substance (other than food) intended to affect the structure or any function of the body. Expansive claims for foods as a cure, mitigation, or prevention of a disease will require extensive clinical trials and scientific proof befitting that of a drug. Furthermore, that food will have to be sold as a drug. This is highly undesirable for a food. Food manufacturers do not have the resources to undergo intense scrutiny of the regulatory authorities, and consumers do not have the wherewithal to pay for foods as drugs. Regulatory authorities are cognizant of the desire of food and commodity manufacturers to make claims for healthy foods and have mechanisms and procedures in place to allow for this.

6.4.1 UNITED STATES FDA REGULATIONS FOR HEALTH CLAIMS (2013)

The 1990 NLEA provides for the FDA to issue regulations authorizing health claims for foods after reviewing and evaluating the scientific evidence either in connection with a submitted health claim or on its own initiative. In 1997, the FDA Modernization Act (FDMA) provided for health claims based on authoritative statement of the National Academy of Sciences or a scientific body of the US government with responsibility for public health protection or nutrition research; such claims may be used 120 days after a health claim notification has been submitted to the FDA, unless the FDA has informed the notifier that the materials submitted do not include all the required information. The FDA reviews the petitions for quality and strength of the submitted evidence, and if it determines that the science supports the claim, they permit the claim; if it does not meet the scientific evidence, it issues a letter of denial for the claim. In many cases, the approved health claim can be used by other manufacturers making the same food and/or using the same ingredients.

A health claim by definition has two essential components, namely, a substance (a food or a food component) and a disease or health condition. A statement lacking either one of these does not meet the regulatory definition of health claim. The FDA provides an example of the role of dietary patterns or of general categories of foods (fruits, vegetables, nuts) in maintaining good health is considered to be dietary guidance rather than a health claim. Dietary guidance used on food labels must be truthful and nonmisleading. Statements that address a role of a specific substance in maintaining normal healthy structures or functions of the body are considered to be structure/function claims. Unlike health claims, dietary guidance and structure/function claims are not subject to premarket review and authorization by the FDA.

Health claims authorized by the NLEA provide for the use in food labeling of health claims that characterize a relationship between a food, a food component or dietary ingredient, and the risk of a disease provided the claim meets certain criteria and are authorized by an FDA regulation. For example, adequate calcium throughout life may reduce the risk of osteoporosis. The FDA authorizes these types of claims after an extensive review of the scientific evidence generally as a result of a submitted
health claim petition. It uses significant scientific agreement to determine whether the substance/disease relationship is well established.

Nutrient content claims pertain to the levels of a particular nutrient in a food product. A claim of “Excellent Source of (name of nutrient)” is permitted if one serving of a product provides 20% of the DV. A claim of “Good Source of (name of nutrient)” can be used if a serving of that product provides between 10% and 19% of the DV for that nutrient. Alternatively, the amount of a particular nutrient can be stated simply as “Contains (percentage of DV) of (name of nutrient).” If the food has been fortified with a particular nutrient such that it is 10% DV higher than a similar product that is not fortified, a claim “Fortified with (name of the nutrient)” is permissible. Many dairy products are “Good” or “Excellent” sources of calcium, phosphorus, potassium, riboflavin, cyanocobalamin, vitamins A and D, and protein. Dairy products can also be modified by removing some components thereby enriching other components. For example, fat in milk can be removed to obtain no-fat milk (commonly referred to as skim milk).

Structure/function claims are another category of health claims. These claims describe the effect of a nutrient or substance on the normal structure or function of the body (e.g., calcium builds strong bones) or on general well-being. Unlike health claims, structure/function claims may be used on food products without prior FDA approval. However, like all other information on a food label, structure/function claims must be truthful and not misleading. For a structure/function claim to be truthful, manufacturers making a claim must have credible scientific evidence to substantiate the claims made.

In a food product if the total fat exceeds 13 g per serving, saturated fat exceeds 4 g, cholesterol exceeds 60 mg per serving, or sodium exceeds 480 mg per serving, a disclosure statement is necessary if nutrient content claims are being made. If levels of any of these nutrients exceed the preceding values, no nutrient content can be made. The disclosure statement “See nutrition information for (nutrient requiring disclosure) content” must appear prominently and in immediate proximity to the claim with no intervening material. If the claim appears on more than one panel, the disclosure statement must accompany it except when the claim appears on the panel that bears the nutrition information, in which case the disclosure statement may be excluded.

For yogurt, “Excellent Source” claims can be made for calcium, riboflavin, and phosphorus and “Good Source” claims for potassium, protein, and zinc. Low-fat plain yogurt can claim “Excellent Source” of calcium, riboflavin, protein, and phosphorus and “Good Source” claim for potassium. Nonfat plain yogurt is an “Excellent Source” of calcium, riboflavin, protein, and phosphorus and a “Good Source” of potassium and zinc. Additionally, fat-free claim can be made for yogurt made with skim milk and light/low-fat claims for yogurt made with 1% and 2% fat milk. Fat-free yogurt can also claim to be free of saturated fats. Low-fat yogurt can also claim to be reduced in saturated fat. Both fat-free and low-fat yogurt can claim to be low in cholesterol.

Structure/function claims recognized by the FDA as qualified health claims are for nutrients involved in maintaining normal healthy functions or structures of the body. These claims may not explicitly or implicitly link the relationship to disease or health-related conditions. Both yogurt and nonfat yogurt are approved by the FDA for calcium and vitamin D and osteoporosis relationships. The wording is “Adequate calcium and vitamin D throughout life, as part of a well-balanced diet, may reduce the risk of osteoporosis” or “Adequate calcium throughout life, as part of a well-balanced diet, may reduce the risk of osteoporosis.” Similarly, for dietary fat and cancer statements for plain and nonfat yogurt, “Development of cancer depends on many factors. A diet low in total fat may reduce the risk of some cancers.” For dietary saturated fats, cholesterol and coronary artery disease a statement
can be made for nonfat yogurt as, “While many factors affect heart disease, diets low in saturated fat and cholesterol may reduce the risk of this disease.” Plain yogurt does not qualify for the coronary artery disease claim because it is high in total fat and cholesterol. Currently, there are no allowed claims for probiotic cultures.

6.4.2 **CODEX ALIMENTARIUS AND HEALTH CLAIMS (CODEX, 1997)**

Codex Alimentarius has not developed standards for nutrition and health claims. The committee charged with labeling activities has, however, drafted guidelines and stated that any claims for nutrients or health must be consistent with national nutrition policy, and Codex supports that policy. The guidelines state that where required by a country advertising materials must be consistent with national nutrition policy. Another guiding principle is that nutrient and health claims shall not be permitted for foods for infants and young children except instances where Codex standards or national legislation specifically provide for such claims.

Codex defines “nutrition claim” as any representation that states, suggests, or implies that a food has particular nutritional properties, including but not limited to, the energy value, protein, fat, carbohydrate, vitamins, or minerals. “Nutrient content claim” is when a food label describes a particular level of a nutrient contained in it. For example, “source of calcium” or “high in fiber” or “low in fat” constitutes a nutrient content claim. “Nutrient comparative claim” is when a food label compares the nutrient value of two or more foods, e.g., “reduced,” “less than,” “fewer,” “increased,” or “more than.” The same guidelines define a “Health claim” as a food label that states, suggests, or implies that a relationship exists between a food, constituent of a food, and health. There can be a nutrient function claim, e.g., nutrient (name) followed by assigning a physiological role for that nutrient in growth, development; food is a source of (nutrient name). Health claims can also be dealing with other functions. Claims that concern a specific beneficial effect of the consumption of foods or their constituents in the context of a total diet on normal functions or biological activities of the body. Such claims are usually positive in their contribution to health or improvement of a function or to modify or preserve health. Lastly, health claims may pertain to reduction of disease risks.

Codex Alimentarius guidelines for nutrient content claims (Table 6.3) are specified. Instead of the US terminology of “daily reference value” (DRV), Codex uses the term “nutrient reference value” (NRV). When a food is by its nature low in or free of the nutrient that is the subject of the claim, the term describing the level of that nutrient should not precede the name of the food but should be in the form “a low (name of nutrient) food” or “a (name of the nutrient)-free food.” Comparative claims should be different versions of the same food, and the foods being compared must be clearly identified. A statement of the amount of difference in the value should be given. The comparison must be based on relative difference of at least 10% NRV.

Codex guidelines state that health claims should be permitted provided the following conditions are met:

1. Health claims must be based on current relevant scientific substantiation, and the level of proof must be sufficient to substantiate the type of claimed effect and the relationship to health.
2. Any health claim must be accepted by the competent authorities of the country where the product is sold.
3. The claimed benefit should arise from the consumption of a reasonable amount of the food or food component in the context of a healthy diet.
4. If the claimed benefit is attributed to a constituent in the food for which an NRV is established, the food in question must be (1) a source of or high in the constituent in the case where increased consumption is recommended or (2) low in, reduced in, or free of the constituent in the case where reduced consumption is recommended. Where applicable the conditions for nutrient content claims and comparative claims will be used (Table 6.3).

5. Only established nutrients for which an NRV has been established in the Codex guidelines or those nutrients that are mentioned in officially recognized dietary guidelines of the national authority having jurisdiction should be the subject of the nutrient function claims.

In summary, Codex Alimentarius has provided guidelines that help national authorities frame nutrient and health claims. It does not prescribe a code that has to be uniformly followed by member countries of the commission.

6.4.3 REST OF THE WORLD

6.4.3.1 European Union (EC, 2006)

This section is by no means an exhaustive survey of the regulations around the globe. Since the Codex Alimentarius has not developed standards for nutrient and health claims, countries have developed their own regulations. The guidelines, however, have been adopted as regulation by the European Union (EU). The EU has tabulated general nutrition and health claims in a register that lists ingredients/compounds, the claim, and the conditions under which the claim can be used. For example, if a food contains calcium the health claim may be for “calcium contributes to normal blood clotting” and/or “calcium contributes to normal energy yielding metabolism.” The blood clotting claim can be used if the food is considered a source of calcium as defined in a 2012 EU Regulation. Similarly, the energy yielding metabolism claim can be used in a food approved as a source of calcium. Calcium also has additional approved claims for “calcium contributes to normal muscle function,” “calcium contributes to normal neurotransmission,” “calcium contributes to normal function of digestive enzymes,” “calcium has a role in the normal cell division and specialization,” “calcium is needed for the maintenance of normal bones,” and “calcium is needed for maintenance of normal teeth.” There are a lot of options for manufacturers to choose from, and if all of them are used for the same food, marketers run the risk of making that food sound more like a nutritional supplement. Live yogurt cultures can claim, “live cultures in yogurt or fermented milk improve lactose digestion in those individuals who have difficulty digesting lactose.” In order to make this claim, the yogurt must contain at least $10^8$ colony-forming units of live starter organisms (L. delbrueckii ssp. bulgaricus and S. thermophilus) per gram. Protein is another nutrient that is important in Greek-style yogurt or concentrated yogurts. If the food is considered a source of protein, several claims are allowed; “protein contributes to muscle mass,” “protein contributes to maintain muscle mass,” and “protein contributes to the maintenance of normal bones” are all permissible by EU regulation. At a minimum, the food must provide 12% of protein per serving.

A 150 g pot of low-fat fruit yogurt provides 13% of daily requirement of protein, which “contributes to the growth and maintenance of healthy bones and muscles.” It also provides 26% of the daily requirement for calcium, which is “needed for growth and maintenance of bones. It also helps muscle and nerve functions, normal blood clotting, and maintenance of healthy teeth.” This pot of yogurt provides 16% of the daily thiamin (vitamin B$_1$), which contributes to “normal nerve and heart function.” Riboflavin (vitamin B$_2$) content is 23% of the daily requirement, which “helps with good skin and
maximizing energy generation from food.” The yogurt in this example also contains 18% of the daily requirement of vitamin B\textsubscript{12}, which “helps to decrease a feeling of tiredness, benefits the immune system, and contributes to the release of energy from the food eaten.” The iodine content in this yogurt is enough for 48% of the daily requirement. Iodine is “good for growth, releasing energy from food, and for good skin.” Yogurt is also a good source of phosphorus, providing 25% of an adult’s daily needs. Phosphorous “helps toward maintenance of healthy bones and teeth.” Last, yogurt provides 15% of the daily needs of potassium, which “contributes to normal muscle and nerve function and aids in the maintenance of healthy blood pressure.”

6.4.3.2 Australia and New Zealand (FSANZ, 2016)

After a long deliberative process, Australia and New Zealand published regulations for nutrient and health claims for food products in 2016. Claims can only be made on food packages that are actually purchased by the consumer. Claims cannot be made for kava, products containing greater than 1.5% alcohol, and infant foods. A nutrient content claim can only be made on a property of food. A descriptor cannot be used in nutrient content claim about lactose or trans-fatty acids unless the descriptor is mentioned in the approved table. Nutrition content claims must not imply slimming effects. Nutrient content claims about choline, fluoride, or folic acid may only state that the food contains them or contains a specific amount of them or a combination of both may be used. A nutrition claim about choline, fluoride, or folic acid can be made only in association with a health claim with regard to the same food. Comparative claims mean nutrition content claim that directly or indirectly compares the nutrition content of one food or brand with another using descriptors such as light (lite), increased, or decreased.

With regard to health claims there are two categories: “general-level health claim” and “high-level health claim.” A health claim is a statement that states, suggests, or implies that a food or a component of the food has an effect or may have an effect on health. Health effect is further clarified to mean an effect on the human body including one or more of the following:

1. a biochemical process or outcome
2. a physiological process or outcome
3. a functional process or outcome
4. growth and development
5. physical performance
6. mental performance
7. a disease, disorder, or condition

High-level health claim means a health claim that refers to a serious disease or a biomarker of a serious disease. A biomarker is defined as a measurable biological parameter that is predictive of a serious disease when present at abnormal levels in the human body. Health claims can only be made if the food meets the Nutritional Profiling Scoring Criterion (NPSC).

Nutrient content claims relating to carbohydrates using the descriptors reduced/light/lite mean that the food has at least 25% less carbohydrate than in the same quantity of a reference food. If the carbohydrate claim in a food pertains to increased carbohydrate, then it must have 25% more carbohydrate than a similar portion of the reference food. Claims relating to cholesterol are similar to the Codex guidelines. To make a general claim for fiber, a serving of the food must contain a minimum of 2 g of fiber. If the serving has 4 g of fiber per serving the claim of “good source” can be made, and if it
contains at least 7 g per serving the food can claim to be an “excellent” source. With respect to energy, “low” energy requires a claim that one serving of a liquid food contains less than 80 kJ per 100 mL or less than 170 kJ per 100 g of solid food. Reduced or lite food must contain at least 25% less energy than in the same quantity of a reference food. The term “diet” can only be used if it meets the NPSC and the energy content is no more than 80 kJ per 100 mL for liquid foods and not more than 170 kJ per 100 g of solid food and contains at least 40% less energy than a similar portion of the reference food. Fat content claims pertain to “% fat” if the food meets the criteria for “low fat.” A “low fat” food contains no more than 1.5 g per 100 mL for a liquid or not more than 3 g per 100 g of a solid food. The term “reduced or light/lite” can be used if the fat content of a serving of the food contains at least 25% less fat than a similar serving of the reference food.

Australia has pioneered measuring the rate of a food’s ability to be converted to glucose. This measure is termed the Glycemic Index (GI), and the higher the value the more glucose that is generated during the digestion of that food. A related term called Glycemic Load (GL) is obtained by multiplying the quantity of carbohydrate in a food by the GI. High GI or GL is indicative of a higher propensity toward adverse health outcomes. GI and GL can be labeled on food packages. If the GI is below 55 the food can be labeled as a “low-glycemic” food; numerical values of GI between 56 and 69 qualify that food to be labeled as “medium glycemic,” and if the food has a GI value of greater than 70 it is considered “high glycemic.” Glycemic load is not listed on the food label. Lactose content of a food can also be labeled. A food containing no detectable amount of lactose can be labeled as “lactose free” and if the lactose content is under 2 g per 100 g it can be labeled as “low lactose.” To label a food as a “good source” of protein it must contain at least 10 g protein per serving. “Increased protein” claim requires a 25% increase in the protein content per serving in comparison to a reference food. Vitamin and mineral content claims (except for potassium or sodium) can be made as a “good source” provided a serving of the food contains at least 25% of the recommended daily intake (RDI) of that vitamin or mineral.

With respect to health claims, high-level health claims for calcium in foods containing no less than 200 mg calcium per serving, a claim of “diet high in calcium enhances bone mineral density” can be used, and if the food also contains vitamin D then “diet high in calcium and adequate vitamin D status reduces risk of osteoporosis or reduces risk of osteoporotic fracture” are permitted. General-level health claims for calcium in foods containing a minimum of 25% of the RDI in a serving of food include the following:

1. Necessary for normal teeth and bone structure
2. Necessary for normal nerve and muscle function
3. Necessary for normal blood coagulation
4. Contributes to normal energy metabolism
5. Contributes to the normal function of digestive enzymes
6. Contributes to normal cell division
7. Contributes to normal growth and development

Iodine is present in milk and yogurt, and claims related to iodine are as follows:

1. Necessary for normal production of thyroid hormones
2. Necessary for neurological function
3. Necessary for normal energy metabolism
4. Contributes to normal cognitive function
5. Contributes to the maintenance of normal skin
6. Contributes to normal growth and development

Phosphorus is another element that dairy foods provide. If a food contributes at least 25% of the RDI for this element, several claims can be made as:

1. Necessary for normal teeth and bone function
2. Necessary for normal cell membrane structure
3. Necessary for normal energy metabolism
4. Contributes to normal growth and development

Among the vitamins that dairy foods provide are riboflavin, thiamin, and B$_{12}$, however, a serving of yogurt normally does not contain at least 25% of the RDI of thiamin and B$_{12}$ and therefore cannot qualify for health claims in Australia and New Zealand. Riboflavin is present in yogurt at just below the 25% RDI threshold and therefore does not qualify for either nutrient content or general-level health claims. Most yogurt products contain live and active cultures. If the level of *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus* is at least $10^8$ cfu/g, then for the individual who has difficulty in digesting lactose, a claim of “improves lactose digestion” is permissible. Health claims for protein are also permissible and may be applicable to concentrated yogurt products but not to regular yogurt. Claims allowed for foods that provide at least 10 g protein per serving can claim the following:

1. Necessary for tissue building and repair
2. Necessary for normal growth and development
3. Contributes to maintenance of muscle mass
4. Contributes to the maintenance of normal bones

### 6.4.3.3 Japan (MHLW, 2016)

In Japan, nutritional labeling on food packages was voluntary until 2015. In 2013, Japan’s Diet (parliament) passed the Food Labeling Act (FLA) to be enforced from 2015 onward. Exemptions from the mandatory labeling of foods include: (1) foods are not important contributors to nutrients (e.g., water and spices), (2) foods that are used as ingredients and will undergo further processing, (3) liquors, (4) foods whose packaging is too small to accommodate the label, (5) foods that are consumed on the same premises as where they are processed, and (6) foods that will be used for schools or hospitals. The nutritional label should provide information on calories, total fat, protein, carbohydrate, and salt. Vitamins and minerals are not a part of the mandatory label. The quantity of salt, instead of sodium content, needs to be shown on the nutrition facts panel. Nutrient content claims cannot be made for foods that contain less than 25% of the standard. This is similar to the guidelines suggested by Codex Alimentarius.

As far as health claims are concerned, there are several types and processes for making claims. First, there is the Foods with Nutrition Function Claims (FNFC), and second, there is the Foods for Specific Health Use (FOSHU). Under FOSHU, the four subtypes are Ordinary FOSHU, Standardized, Reduction of Disease Risk, and Qualified. Beginning with FNFC, there are only 12 vitamins and 5 minerals that qualify under this claim. The vitamins are niacin, pantothenic acid, biotin, vitamin A, vitamin B$_{1}$, vitamin B$_{2}$, vitamin B$_{6}$, vitamin B$_{12}$, vitamin C, vitamin D, vitamin E, and folic acid. The five minerals are zinc, calcium, iron, copper, and magnesium. If a food contains at least 25% of the reference amounts of any of these 17 ingredients, good source claims may be made of the ingredients
that qualify. The health connection between these 17 nutrients comes with the exact wording for the
claim and the warning that must accompany such a claim. Using the example of calcium, which has an
advisable daily intake of 240–600 mg, the claim that is permissible is “Necessary for development of
bones and teeth” and must be accompanied with a warning that says “Increased intake of this product
will not result in curing diseases nor promoting health. Please comply with the advisable intake.”

Ordinary FOSHU applies to instances where nutrients may have an effect on the physiology of
humans. When foods are sold for a specific health use, the government agency has to critically assess
the scientific validity of the claim and the food manufacturer has to have approval for that food.
Standardized FOSHU claim involves no detailed review of foods that have been ruled as meeting scien-
tific basis for a claim. For efficiency of the approval process, products that have been approved after
safety and scientific review need not apply for permission. Health functions with approved health claims
under FOSHU are gastrointestinal health, cholesterol moderation, hypertension moderation, lipid
metabolism moderation, sugar absorption moderation, mineral absorption, bone health, and tooth health.

Reduction of risk FOSHU involves a detailed scientific review of every product, and is permitted for
food products or ingredients clinically and nutritionally established to reduce the risk of a certain dis-
ease like calcium and osteoporosis. Qualified FOSHU claims require detailed scientific review of evi-
dence in each instance and consist of foods containing ingredients that do have some effect on health
but do not stand up to the rigor of standards set for FOSHU. Such foods have to be labeled as “Qualified
Food for Specific Health Use.” Japan also has a category titled Food for Specific Dietary Uses (FOSDU).
Individual foods for medical conditions fall under this category. Low-sodium foods, low-calorie foods,
low-protein foods, low (no)-protein high-calorie foods, high-protein foods, allergen-free foods, lactose-
free foods, low-sodium adjusted compounded foods, diabetes adjustment compounded foods, liver dis-
ease adjustment compounded foods, and adult obesity adjustment compounded foods all form a part of
FOSDU. In 2012, fully 70% of foods with FOSHU status were concerned with the gastrointestinal tract.

6.5 SUMMARY

This chapter aimed to provide a bird’s eye view of the laws and regulations in select regions in the
world. It is not meant to be exhaustive nor to delineate every nuance of a regulation. Further, the food
safety aspects of manufacturing dairy products are not considered in detail. However, that is a very
important aspect of any food manufactured for human consumption. Since the author is familiar to a
greater degree with regulations of the United States, that section may appear to be larger than those of
other parts of the world.

Basically, the regulations promulgated by the FDA have been extensively revised with the imple-
mentation of the Food Safety Modernization Act. In many other countries regulations promulgated by
the Codex Alimentarius have been adopted. The purpose of the Codex Alimentarius is to harmonize
regulations between nations so that trade can be conducted more easily. The US regulations are in com-
pliance with the Codex standards. Perhaps the greater area of diversity resides in regulations and guide-
lines pertaining to nutritional labeling, nutrient content claims, and health claims. Codex guidelines
have been accepted by the countries of the EU and many other countries. Some other countries have
modified Codex guidelines, and in other countries there are no provisions for nutrient content claims or
health claims. Companies interested in exporting products are required to check the laws and regula-
tions of the country receiving these products.
REFERENCES


PART

YOGURT ADDITIVES AND REFORMULATIONS
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OMEGA-3 POLYUNSATURATED FATTY ACIDS ADDED TO YOGURT

7.1 OMEGA-3 FATTY ACID CLASSIFICATION AND STRUCTURE

Fatty acids are carboxylic acids with a variable length aliphatic hydrocarbon chain. Carbon atoms in the hydrocarbon chain are numbered starting at the carboxylic acid end when using the systematic, or International Union of Pure and Applied Chemistry (IUPAC), nomenclature. The ω (omega) letter refers to the last carbon atom (the carbon atom in the terminal methyl group) in a fatty acid when using the IUPAC rules for numbering carbon atoms. Alternatively, carbon atoms are numbered starting from the terminal methyl carbon atom when using the n-x nomenclature. Additional details about naming fatty acids can be found at https://en.wikipedia.org/wiki/Fatty_acid.

Fatty acids are classified as saturated, monounsaturated, and polyunsaturated (PUFA). The location of the double bond in unsaturated fatty acids can be specified using either the C-n notation (carbon numbering based on IUPAC rules) or the ω-n notation (carbon numbering based on the n-x nomenclature). Different types of omega unsaturated fatty acids exist including omega-3 fatty acids (also known as ω-3 or n-3 fatty acids), omega-6 fatty acids (also known as ω-6 or n-6 fatty acids), omega-7 fatty acids (also known as ω-7 or n-7 fatty acids), and omega-9 fatty acids (also known as ω-9 or n-9 fatty acids).

Using the n-x carbon numbering system for specifying location of the first carbon atom having a double bond but retaining the ω notation derived from IUPAC, omega-3 fatty acids are PUFA$s in which the first carbon–carbon double bond occurs between the third and fourth carbon atoms and with one methylene (CH₂) group before the next carbon–carbon double bond. Therefore, the second carbon–carbon double bond occurs between the sixth and seventh carbon atoms. Likewise, the third carbon–carbon double bond occurs between the ninth and tenth carbon atoms. Additional carbon–carbon double bonds may also be present closer to the carboxyl group. The carbon–carbon double bonds are in the cis-configuration (meaning that both hydrogen atoms bonded to these two carbon atoms are on the same side of the double bond). The common omega-3 fatty acids are listed at https://en.wikipedia.org/wiki/Omega-3_fatty_acid. The ω-linolenic acid (ALA, 18 carbons with 3 double bonds) (Fig. 7.1), eicosapentaenoic acid (EPA, 20 carbons with 5 double bonds) (Fig. 7.2), and docosahexaenoic acid (DHA, 22 carbons with 6 double bonds) (Fig. 7.3) omega-3 fatty acids are the ones that are most important in human nutrition and physiology. Docosapentaenoic acid (DPA, 22 carbons with 5 double bonds) is structurally similar to EPA except for an extra two carbons in the chain.

Omega-6 fatty acids are PUFA$s in which the first carbon–carbon double bond occurs between the sixth and seventh carbon atoms when using the same carbon numbering system as used for omega-3
fatty acids. Similar to omega-3 fatty acids, omega-6 fatty acids typically have one methylene (—CH₂—) group before the next carbon–carbon double bond and the carbon–carbon double bonds are in the cis-configuration. The most recognized, as well as having the shortest chain, omega-6 fatty acid is linoleic acid (18 carbons with 2 double bonds). A list of omega-6 fatty acids is given at https://en.wikipedia.org/wiki/Omega-6_fatty_acid.

Omega-7 fatty acids and omega-9 fatty acids are other types of unsaturated fatty acids and are not as well recognized by many as omega-3 fatty acids. The first carbon–carbon double bond occurs between the seventh and eighth carbon atoms in omega-7 fatty acids and between the ninth and tenth carbon atoms in omega-9 fatty acids. Omega-7 fatty acids have one carbon–carbon double bond. Palmitoleic acid (16 carbon atoms with 1 double bond) and vaccenic acid (18 carbon atoms with 1 double bond) are the two most common omega-7 fatty acids, and a list of omega-7 fatty acids is provided at https://en.wikipedia.org/wiki/Omega-7_fatty_acid. Important omega-9 fatty acids include oleic acid (18 carbon atoms with 1 double bond) and erucic acid (22 carbon atoms with 1 double bond), and a list of omega-9 fatty acids is provided at https://en.wikipedia.org/wiki/Omega-9_fatty_acid.

7.2 SOURCES OF OMEGA-3 FATTY ACIDS

Sources of omega-3 fatty acids include fatty fish and various plant sources. Fatty fish, especially anchovy, herring, salmon, and mackerel, is rich in EPA and DHA and these levels are presented by Mozaffarian and Wu (2011). DPA is normally present in lesser amounts than EPA and DHA in fish (Mozaffarian and Wu, 2011). Plant sources such as nuts and seeds (especially flaxseed), vegetables,
legumes, grains, and fruit contain ALA, and the amounts in these sources are presented by Mozaffarian and Wu (2011) and Kris-Etherton et al. (2000). In the Estrada et al. (2011) study, PUFAs constituted 32.72% of the total fatty acids in microencapsulated salmon oil used in their study, and 62.6% of these PUFAs were either EPA or DHA. The total omega-3 fatty acid content in their microencapsulated salmon oil was 29.48%. Nielsen et al. (2007) reported molar percentages of 1.3% of 18:3 (n-3), 2.7% of 18:4 (n-3), 8.5% of 20:5 (n-3), and 11.0% of 22:6 (n-3) in fish oil.

### 7.3 OXIDATION OF POLYUNSATURATED FATTY ACIDS AND OMEGA-3 FATTY ACIDS

A problem with polyunsaturated fatty acids including omega-3 fatty acids is oxidation. Long-chain omega-3 PUFA can oxidize to form toxic products such as peroxides and volatile compounds leading to off-flavors (Rubio-Rodríguez et al., 2010). Some volatile oxidation compounds that can form include 1-penten-3-ol, 1-penten-3-one, (E)-2-pentenal, (E)-2-hexenal, (E,E)-2,4-heptadienal, and (E,Z)-2,6-nonadienal (Let et al., 2007).

Oxidation can be quantified in several ways. These methods include peroxide values and inhibition of lipid peroxidation in liposome model system, anisidine values, free fatty acid content, thiobarbituric acid value, scavenging of 1,1-diphenyl-2-picrylhydrazyl free radical, reducing power, and iron ion chelation capacity. Initial lipid oxidation can be measured by peroxide value. Peroxide values in the Estrada et al. (2011) study were 10.13, 5.16, 6.32 meq/kg for unpurified salmon oil, purified salmon oil, and microencapsulated salmon oil, respectively. Anisidine values measure secondary oxidation products. In the Estrada et al. (2011) study, anisidine values for unpurified salmon oil, purified salmon oil, and microencapsulated salmon oil were 0.43, 0.58, and 0.72, respectively. Free–fatty acid content decreased from 1.61% for unpurified salmon oil to 0.61% for purified salmon oil but rose to 0.77% for microencapsulated salmon oil.

### 7.4 GENERAL HEALTH BENEFITS OF OMEGA-3 FATTY ACIDS

Early work that stimulated interest in health benefits of fish oil consumption was a study relating high consumption of seal and fish by Eskimos from northwestern Greenland likely to explain their low rates of death from ischemic heart diseases (Bang et al., 1980). The diets of these Eskimos contained a high average daily intake of cholesterol (264 mg/1000 kcal), reduced saturated fatty acid content, and increased monounsaturated and polyunsaturated fatty acid contents including EPA, DPA, and DHA. These Eskimos had low levels of triglycerides, cholesterol, low-density lipoproteins (LDLs), and very low-density lipoproteins but increased high-density lipoproteins (HDLs) in their plasma. In addition to only 3.5% of all deaths in Greenland Eskimos being due to ischemic heart diseases, Bang et al. (1980) reported that Eskimos are rarely obese and rarely have hypertension or diabetes mellitus.

Much research has continued to be published dealing with effects of omega-3 fatty acids on the cardiovascular system. Omega-3 fatty acid prescription formulations appear to be effective in treating patients with severe hypertriglyceridemia (Backes et al., 2016). Some additional cardiovascular benefits provided by omega-3 fatty acid consumption include lowered resting heart rate and blood pressure and may include improved myocardial filling and efficiency, lowered inflammation, and improved
vascular function as summarized by Mozaffarian and Wu (2011). Consumption of 2 g/day of fish oil omega-3 fatty acids resulted in 15% lower triacylglycerol levels (Khandelwal et al., 2009), no significant change in total cholesterol (Khandelwal et al., 2009), increased HDL cholesterol by 5.4% (Khandelwal et al., 2009), a nonsignificant (3.8%) increase in non-HDL cholesterol (Khandelwal et al., 2013), no change in LDL cholesterol (Khandelwal et al., 2009, 2013), no changes in LDL particle size (Khandelwal et al., 2009), no decrease in apoB concentration and no increase in apoA1 concentration (Khandelwal et al., 2009). After pooling the data from 19 cohort studies, Del Gobbo et al. (2016) concluded that biomarker concentrations of omega-3 fatty acids are related to a modestly lower number of cases of fatal coronary heart disease.

Skeletal muscle mass lost during aging, called sarcopenia, leads to an increased risk of chronic disease in addition to frailty and reduced quality of life (Jeromson et al., 2015). Smith et al. (2011) has shown that muscle protein synthesis in older adults is stimulated by omega-3 fatty acids. Jeromson et al. (2015) summarized possible molecular mechanisms by which omega-3 fatty acids may affect skeletal muscle, including the omega-3/omega-6 fatty acid ratio, inflammation, and lipidomic remodeling.

Another area of omega-3 fatty acid research is for arthritis. Active rheumatoid arthritis can be alleviated by ingestion of fish oil dietary supplements (Kremer et al., 1987). However, Boe and Vangsness (2015) concluded that the clinical evidence needed for justifying use of fish oils for treating or preventing osteoarthritis appeared to be insufficient.

Omega-3 fatty acids are also important in vision. Taking an oral supplement containing omega-3 fatty acids, vitamins, minerals, and antioxidants was effective for treating dry eye symptoms (Gatell-Tortajada, 2016). These dry eye symptoms included scratchy and stinging sensation, eye redness, grittiness, painful and tired eyes, grating sensation, and blurry vision. Christen et al. (2011) found that the risk of age-related macular degeneration in women was significantly reduced with regular consumption of DHA and EPA. The antiinflammatory effects of omega-3 fatty acids have been discussed in being involved in their beneficial effects for both dry eye symptoms and age-related macular degeneration (Gatell-Tortajada, 2016; Christen et al., 2011) and the importance of both the omega-3 fatty acid intake and the omega-6 to omega-3 fatty acid ratio in determining the risk for developing age-related macular degeneration was noted (Christen et al., 2011).

Long-chain omega-3 fatty acids are important for mental health and cognitive functioning. Messamore and McNamara (2016) reported that blood EPA plus DHA levels being 4% or less of the total fatty acid composition occurred in 75% of psychiatric patients compared to 25% of the normal population. Mocking et al. (2016) concluded that there was an overall beneficial effect of omega-3 fatty acid supplementation in patients with major depressive disorder, especially when patients consumed high EPA doses and took antidepressants. Su et al. (2015) concluded that the regulatory effects of omega-3 fatty acids on immunomodulation, antiinflammation, signal transduction, neurotransmission, and neuroprotection support the clinical evidence of their beneficial preventive effects on mood and anxiety disorders. Children between 8 and 16 years old consumed either a fruit drink containing 1 g of omega-3 fatty acids daily for 6 months or a placebo in the study of Raine et al. (2015). The children in the omega-3 fatty acid group had a 42%–68% reduction in parent-reported externalizing and internalizing behavioral problems. Improvement in behavior continued for 6 months after stopping treatment (Raine et al., 2015). Layé et al. (2015) reported that omega-3 fatty acids play a role in the development of age-linked neurodegenerative disorders such as Alzheimer disease as shown by epidemiological studies. Bloch and Qawasmi (2011) performed a meta-analysis of treating children with attention
deficit hyperactivity disorder (ADHD) with omega-3 fatty acid supplementation. They found a small, but significant, effect for improving ADHD symptoms. Also, they found EPA dose within supplements and supplement efficiency were significantly correlated. D’Ascoli et al. (2016) reported an association for older men and women between higher serum long-chain omega-3 fatty acid concentrations and better performance on neuropsychological tests of frontal lobe functioning.

Much research has been done on effects of omega-3 fatty acids on maternity and fetal development. Le Donne et al. (2016) divided mothers who recently gave birth into a fish-consuming group and a nonfish-consuming group. They found that the nonfish-consuming group tended to have a higher frequency of pregnancy-induced hypertension. Also, there was a positive correlation between consumption of small oily fish with both neonatal weight and head circumference (Le Donne et al., 2016). Calabuig-Navarro et al. (2016) found decreased fatty acid esterification and a lower total lipid concentration in placentas of overweight and obese women who took supplemental omega-3 fatty acids compared to those who took a placebo. Also, they found a higher birth weight and length of the offspring from the women who took supplemental omega-3 fatty acids compared to the placebo. Vidakovic et al. (2016) found an association between a higher maternal plasma omega-3 fatty acid concentration with both a decreased childhood total body fat percent and a lower android-gynoid fat mass ratio. Maternal intake of DHA during pregnancy has been shown to improve the mental processing of their children at four years of age (Helland et al., 2003).

DPA consumption also leads to health benefits. Byelashov et al. (2015) reported that increased blood DPA levels are positively correlated with reduced blood triglycerides, cholesterol, inflammation, and overall risk of coronary heart diseases and acute myocardial infarction. Improved mental health and cognitive function have also been related to higher DPA levels (Byelashov et al., 2015).

Although ALA is also an essential dietary fatty acid and might provide some cardiovascular health benefits, the overall evidence of health benefits is not conclusive (Mozaffarian and Wu, 2011). Even though there are biochemical pathways that convert ALA to EPA and EPA to DHA, this conversion in humans is limited by between 0.2% and 8% conversion of ALA to EPA and between 0% and 4% conversion of ALA to DHA (Mozaffarian and Wu, 2011).

### 7.5 RECOMMENDED INTAKES AND RATIO OF OMEGA-6 TO OMEGA-3 FATTY ACIDS

The 2015–2020 Dietary Guidelines (U.S. Department of Health and Human Services and U.S. Department of Agriculture, 2015), the European Food Safety Authority (EFSA) Panel on Dietetic Products, Nutrition, and Allergies (EFSA NDA Panel, 2010), and the subcommittee on Recommendations for Intake of Polyunsaturated Fatty Acids in Healthy Adults (International Society for the Study of Fatty Acids and Lipids, 2004) have made various recommendations for daily consumption for omega-3 fatty acids. The 2015–2020 Dietary Guidelines recommend consuming 8 oz of a variety of seafood weekly supplying an average daily consumption of 250 mg of EPA and DHA to reduce cardiac deaths both in individuals with and without preexisting cardiovascular disease. Also, these guidelines recommend that pregnant or breastfeeding women consume between 8 and 12 oz of seafood per week as a source of DHA for improving infant health. The EFSA NDA Panel has recommended intakes of 0.5% of the total energy as an adequate intake for ALA but no tolerable upper intake level. Adequate intake of EPA plus DHA has been proposed at 0.25 g based on cardiovascular considerations. During
pregnancy, an extra 0.1–0.2 g of preformed DHA should be consumed. DHA should be from 50 to 199 mg for older infants. Children should consume 0.25 g of EPA plus DHA per day, similar to adults (EFSA NDA Panel, 2010). The International Society for the Study of Fatty Acids and Lipids has recommended an adequate intake of 2% of the energy from linoleic acid, a healthy intake of 0.7% of the energy from ALA, and a minimum combined intake of 500 mg/day for EPA and DHA.

The ratio of omega-6 fatty acids to omega-3 fatty acids is important. Although a balanced omega-6 to omega-3 fatty acid ratio is 1–2/1, this ratio has changed from 1:1 during evolution to at least 20:1 today in typical Western diets (Simopoulos, 2016). Omega-6 and omega-3 fatty acids compete for desaturation enzymes, so the desaturation and elongation of ALA is interfered with a high linoleic acid intake. This high omega-6 to omega-3 fatty acid ratio is highly prothrombotic and proinflammatory, leading to increased prevalence of atherosclerosis, obesity, and diabetes. Therefore, an increased intake of omega-3 fatty acids and a decreased intake of omega-6 fatty acids to obtain a balanced omega-6 to omega-3 fatty acid ratio are recommended to prevent obesity.

### 7.6 CONSUMPTION OF OMEGA-3 FATTY ACIDS

Surveys have been conducted to quantify consumption of omega-3 fatty acids in the United States and in Australia. In an earlier study, Kris-Etherton et al. (2000) reported that the total omega-3 fatty acid consumption in the United States is approximately 1.6 g/day. This includes 1.4 g of ALA and 0.1–0.2 g of EPA and DHA. A more recent report found mean usual intake of 1.6 g/day of ALA, 41 mg/day of EPA, and 72 mg/day of DHA from foods and dietary supplement use for adults in the United States at least 19 years old (Papanikolaou et al., 2014). Meyer (2016) reported that adult Australians consume a mean of 395 mg/day (consisting of 277 mg from food and 118 mg from supplements) of long-chain omega-3 fatty acids (EPA, DPA, and DHA).

Although an average of 78% of adults in the United States, Germany, and United Kingdom believe that omega-3 fatty acids are important for overall health (Bailey et al., 2015), surveys find that most people do not consume a sufficient amount of omega-3 fatty acids. Meyer (2016) reported only 20% of the Australian population consumes the recommended amount of long-chain omega-3 fatty acids and 10% of women of childbearing age consumes the recommended amount of DHA needed for pregnancy. Likewise, Papanikolaou et al. (2014) have stated that a large percentage of adults in the United States does not consume a sufficient amount of omega-3 fatty acids. Drewery et al. (2016) reported that pregnant women in Louisiana are not likely meeting their long-chain omega-3 fatty acid requirements because of their low seafood consumption as determined by an observational study.

### 7.7 LEVELS OF OMEGA-3 FATTY ACIDS IN MILK AND UNFORTIFIED YOGURT

Levels of omega-3 fatty acids in milk are low. Collomb et al. (2004) found EPA, DPA, and DHA fatty acid percentages in milk fat of 0.07%, 0.10%, and 0.01%, respectively, and a total omega-3 fatty acid (C18:2 t11c15, C18:2 c9c15, ALA, C20:3, EPA, DPA, and DHA) concentration of 1.1% from cows fed a control diet of hay and fodder beet. The total omega-3 fatty acid percent can be increased, largely by increasing ALA percent, by feeding cows ground linseed, which contains a high concentration of ALA (Collomb et al., 2004). Naydenova et al. (2013) found that the C18:3 (ALA) fatty acid percent of the
total fatty acids increased from 0.65% in raw buffalo milk to 0.77% in yogurt made from this milk, and the omega-6 to omega-3 fatty acid ratio decreased from 4.30 in raw buffalo milk to 3.58 for yogurt made from this milk. EPA and DHA were not detected in a strawberry yogurt control (not containing added microencapsulated salmon oil) (Estrada et al., 2011).

### 7.8 INCREASING OMEGA-3 FATTY ACID CONTENT

#### 7.8.1 FEEDING COWS FISH OIL

One way to increase omega-3 fatty acid content in milk is to feed fish oil to cows. However, feeding fish oil to cows decreases milk fat secretion. Chilliard et al. (2001) calculated a transfer efficiency (increase of milk secretion of EPA and DHA compared to the control as a percentage of increase in intake of EPA and DHA compared to the control) of 2.6% for EPA and 4.1% for DHA obtained from data from numerous studies. DPA has higher transfer efficiency (30%–39%) (Chilliard et al., 2001). Dave et al. (2002) also found decreased milk fat content but an increased concentration of omega-3 fatty acids in the milk fat upon feeding a diet containing added fish oil to cows. The omega-3 fatty acid concentration in the Dave et al. (2002) study was not significantly affected by incorporation of milk powder to make the yogurt mix, heat treatment of this mix at 85°C for 30 min, fermentation with yogurt starter and probiotic bacteria, and storage of this yogurt.

#### 7.8.2 FORTIFYING FOODS WITH FISH OIL

Another way to increase the level of omega-3 fatty acids in foods and to develop a more desirable ratio between omega-3 fatty acids and omega-6 fatty acids is to fortify various types of foods with omega-3 fatty acids or by replacing some of the original fat in products with fish oil. Many types of foods have been fortified with fish oil. A list of commercial fortified bread and bakery products, milk and dairy products, spreadable fats, egg and egg products, meat and poultry products, and juices and soft drinks is provided by Kolanowski and Laufenberg (2006).

#### 7.8.3 ADDITION OF FISH OIL TO YOGURT

Fish oil has been added to yogurt to increase the omega-3 fatty acid content in this product. Let et al. (2007) stated that yogurt appeared to be a suitable product for delivering fish oil, and Nielsen et al. (2007) reported that yogurt can be a desirable product for fish oil enrichment because of the healthy image and widespread consumption of yogurt. Rognlien et al. (2012) stated that omega-3 fatty acid levels in commercially available yogurts fortified with these lipids are 20–60 mg per serving. Although the risk of off-flavors or odors at these levels is minimized, many servings need to be consumed to meet the recommended daily intake. The 0.25% fish oil enriched yogurt in the study of Risé et al. (2011) contained 1.91% EPA (wt/wt) and 2.22% DHA (wt/wt) of the total weight of fatty acids.

Different methods exist for adding omega-3 fatty acids to yogurt. Yogurt has been supplemented directly with neat fish oil. Directly adding DHA or EPA to foods usually leads to a fishy odor and taste because of oxidation of these fatty acids (Estrada et al., 2011). Another method of adding fish oil to yogurt is to form a stabilized preemulsion of the omega-3 PUFA oil before addition to the yogurt, so only gentle agitation, including hand stirring, of this preemulsion into yogurt would be sufficient.
instead of requiring additional emulsification (Let et al., 2007). An advantage of this type of addition is that fish oil can be added late in the process to minimize the contact with potential prooxidative compounds resulting in decreased exposure to light, heat, and oxygen (Let et al., 2007). Chee et al. (2005) formed an algae oil emulsion for providing omega-3 fatty acids to yogurt.

To decrease oxidation, potentially unstable materials such as fish oil are packed into a film to form microencapsulated particles. Microencapsulation of marine lipids commonly involves spray-drying of emulsions. Large amounts of surface oil, low oil content, and instability limit the commercial use of spray-dried emulsions (Tamjidi et al., 2012). However microencapsulated fish oil made by a spray-dried complex coacervate has recently been incorporated into many food products (Tamjidi et al., 2012). Tamjidi et al. (2012) prepared fish oil microcapsules by emulsifying fish oil in a gelatin solution before adding an acacia gum solution. The microencapsulated salmon oil in the Estrada et al. (2011) study was prepared by forming an emulsion consisting of 7% purified salmon oil, 22% gum arabic, 11% maltodextrin, and 60% water and spray-drying this emulsion.

Many authors have successfully added microencapsulated oil containing omega-3 fatty acids to yogurt. Estrada et al. (2011) added microencapsulated salmon oil to dry yogurt ingredients before homogenization and pasteurization to develop a strawberry yogurt containing 2% (weight/volume) microencapsulated salmon oil. Addition of microencapsulated salmon oil during the manufacture of strawberry yogurt raised the total omega-3 fatty acid content from 1.76% to 7.50% of the total fatty acids and the EPA and DHA contents from undetectable to 2.9% and 2.37%, respectively, of the total fatty acids. However, the total omega-3 fatty acid, EPA, and DHA contents each decreased during 4 weeks of storage in their study. Tamjidi et al. (2012) did not detect fish oil release from microcapsules using gas chromatography in oil extracted from yogurt containing microencapsulated fish oil. Liu et al. (2016) encapsulated fish oil into polymerized whey protein and incorporated these particles into low-fat yogurt as a fat replacer, and the microencapsulation protected the fish oil from oxidation and its fishy smell.

Comparisons of oxidation of fish oil–enriched milk and fish oil–enriched yogurt and their method of incorporation of the fish oil have been made. Yogurt enriched with fish oil had lower levels of a fishy off-flavor and oxidized slower than milk enriched with fish oil (Let et al., 2007). Enriching yogurt with neat fish oil rather than a fish-oil-in-water emulsion resulted in a more stable product. Let et al. (2007) found higher peroxide values and higher levels of volatile oxidation products for yogurt enriched with fish-oil-in-water emulsion versus yogurt enriched with neat fish oil after 29 days of storage. Also, there was a nonsignificant tendency for increasing fishy off-flavor in the yogurt enriched with fish-oil-in-water emulsion during storage (Let et al., 2007).

Much research has been conducted to measure and to attempt to minimize lipid oxidation and rancidity in yogurt and drinking yogurt fortified by omega-3 fatty acids. Chee et al. (2005) supplemented a yogurt mix with an algae oil emulsion either immediately before or immediately after homogenization and found similar rates of formation of lipid hydroperoxides during storage of these two types of strawberry-flavored yogurts, indicating that homogenization did not affect lipid oxidation. Farvin et al. (2010) reported that fish-oil-enriched yogurt has a high-oxidative stability because of the antioxidative activity of the peptides that are released during milk fermentation by lactic acid bacteria to produce yogurt and because of a reduced oxygen content in yogurt compared to milk. Molecular weight fractions of 3–10 kDa and less than 3 kDa provide much of this increase in oxidative stability compared to higher molecular weight fractions derived by separation by ultrafiltration of crude yogurt supernatant (Farvin et al., 2010). Drinking yogurt enriched with fish oil was very stable to oxidation as yogurt stored at 2°C for up to 4 weeks did not undergo oxidation, and it was more stable to oxidation than milk...
enriched with fish oil probably due to the lower pH of the drinking yogurt. Oxidation of drinking yogurt was not promoted with the addition of 50 μM iron. The volatile compound, hexanal, in drinking yogurt decreased during 4 weeks of storage, while there was no change in trans, cis-2,4-hexadienal. Free fatty acid content in drinking yogurt increased from 5.58% at the start of storage to 6.11% after 6 days of storage at 2°C (Nielsen et al., 2007). Nielsen et al. (2007) reported peroxide values of drinking yogurt containing fish oil changing from 2.21 meq/kg at week 0 to 2.35 meq/kg at week 4 of storage at 2°C. Encapsulation reduced omega-3 fatty acid oxidation as Tamjidi et al. (2012) reported peroxide values of 1.25 meq O₂/kg on the 10th day of storage and 1.72 meq O₂/kg on the 22nd day of storage for encapsulated fish oil yogurt and peroxide values of 2.35 meq O₂/kg on the 10th day of storage and 3.60 meq O₂/kg on the 22nd day of storage for yogurt containing free fish oil. The presence of microencapsulated salmon oil in strawberry yogurt increased thiobarbituric acid values, and the thiobarbituric acid values increased during storage in the Estrada et al. (2011) study. To prevent formation of peroxides and other harmful products from lipid oxidation of omega-3 PUFA, foods fortified with fish oil should be manufactured, packaged, stored, and distributed minimizing exposure to oxygen, UV light, high temperature and humidity, and metallic ions especially iron, copper, and manganese (Kolanowski and Weiβbrodt, 2007).

Sensory results have been mixed because high levels of fish oil fortification may adversely affect its sensory properties during storage because of development of unacceptable fish oil off-flavor and susceptibility to oxidative deterioration. Rognlien et al. (2012) reported that low levels of fortification of yogurt with fresh fish oil are acceptable. Unflavored yogurt containing 0.5% wt/wt of fish oil or 0.5% wt/wt of butter oil could not be differentiated by panelists in the study of Rognlien et al. (2012). Likewise, oxidized flavor perception of a chile-lime-flavored yogurt containing 0.43% fish oil could not be distinguished from yogurts containing butteroil at levels of 0.43% or 1%. Also, fortification of a chile-lime-flavored yogurt was acceptable to a portion of panelists. However, fortification of yogurt with oxidized fish oil, even at the low level of 0.43%, can be readily detected by panelists. Also, fortification of yogurt with a high level (1%) of oxidized fish oil will lead to a decreased perception of lime and acid flavors in chile-lime-flavored yogurt (Rognlien et al., 2012). Although Tamjidi et al. (2012) found no significant differences for appearance and texture scores on a 9-point hedonic scale between yogurt containing microencapsulated fish oil and a control yogurt, aroma, flavor, and overall acceptance scores were significantly lower for yogurt containing microencapsulated fish oil than the corresponding scores for their control. However, the aroma, flavor, and overall acceptance scores were significantly improved, but still significantly lower than the control yogurt, by treating microcapsules with diluted lime juice for 24 h before adding it to the milk. Kolanowski and Weiβbrodt (2007) reported highest fortified fish oil levels without adversely affecting overall sensory quality (threshold values) of 2 and 3 g/kg of fish oil addition to a 2% fat unflavored drinkable yogurt and a 2% fat drinkable yogurt flavored with strawberry, respectively. In the study of Chee et al. (2005), the increased fishy perception by a trained sensory panel for strawberry-flavored yogurt fortified with an algae oil emulsion added either immediately before or immediately after homogenization relative to the control (without algae oil emulsion) was occasionally statistically significant. However, only very minor differences in overall liking scores rated on a 9-point hedonic scale for strawberry-flavored yogurt fortified with an algae oil emulsion added to the milk and their control were found, and these samples were evaluated as “liked moderately.” Both Kolanowski and Weiβbrodt (2007) and Chee et al. (2005) showed that flavorings such as strawberry can aid in masking the off-flavors that develop due to fish oil fortification. Additionally, Chee et al. (2005) suggested that the strong antioxidative properties of milk proteins, especially casein, may explain the relatively
slow rate of oxidation of omega-3 fatty acids in yogurt. Radulovic et al. (2013) reported acceptable sensory properties of yogurt containing Lactobacillus paracasei 08 and long-chain omega-3 fatty acids at levels of 100 and 200 mg/L during 21 days of storage.

Microbial counts have been enumerated in yogurt fortified with omega-3 fatty acids. Long-chain omega-3 fatty acids at levels of 100 and 200 mg/L did not significantly affect counts of Lactobacillus delbrueckii subsp. bulgaricus, Streptococcus thermophilus, and L. paracasei 08 during 21 days of storage (Radulovic et al., 2013). Lactic acid bacteria counts in strawberry yogurt containing microencapsulated salmon oil were slightly higher than strawberry yogurt without salmon oil during the first week of storage but were not significantly different during subsequent storage (Estrada et al., 2011).

The pH and titratable acidity have been reported in studies of yogurt fortified with omega-3 fatty acids. Radulovic et al. (2013) reported similar pH changes during 21 days of storage for yogurt containing L. paracasei 08 and omega-3 fatty acids at levels of 100 and 200 mg/L during 21 days of storage. Microencapsulated salmon oil addition to yogurt did not affect yogurt pH in the Estrada et al. (2011) study. Likewise, Tamjidi et al. (2012) reported no significant difference in pH of yogurt containing microencapsulated fish oil compared to its control during 21 days of storage but a slight increase in titratable acidity of yogurt containing microencapsulated fish oil compared to its control, probably due to the acidification of the added fish oil powder during encapsulation.

Physical properties including serum separation, water-holding capacity, firmness, and viscosity of yogurt fortified with omega-3 fatty acids have also been studied. Tamjidi et al. (2012) found significantly less serum separation as measured by amount of clear supernatant that can be poured off after centrifugation at 222×g for 10 min and significantly higher water-holding capacity in yogurt containing microencapsulated fish oil powder than for their control yogurt for 21 days of storage. The capability of the whole gel structure of strawberry yogurt to retain water as measured by funnel drainage was generally not significantly affected by addition of microencapsulated salmon oil in the Estrada et al. (2011) study. Tamjidi et al. (2012) found that yogurt containing microencapsulated fish oil was significantly less firm between 7 and 21 days of storage, but not at 1 day of storage, than the control yogurt, but the apparent viscosity of the yogurt containing microencapsulated fish oil was significantly higher than the apparent viscosity of the control yogurt. Tamjidi et al. (2014) reported that this increased apparent viscosity occurred at each shear rate, but the apparent viscosity decreased more for the yogurt containing microencapsulated fish oil than for the control yogurt when increasing the shear rate, regardless of when the viscosity was measured during the storage time. This greater decrease in apparent viscosity can be explained by faster protein aggregate disruption in the yogurt containing microencapsulated fish oil (Tamjidi et al., 2014). Let et al. (2007) reported higher apparent viscosity of yogurt containing neat fish oil versus yogurt containing fish-oil-in-water emulsion initially, but these viscosities became similar during later stages of storage. A value of 0.0136 Pa/s for viscosity of drinking yogurt containing fish oil has been reported by Nielsen et al. (2007).

Oil droplet sizes have also been reported. Let et al. (2007) found that average oil droplet sizes increased from 5.0 μm at day 1 of storage to 6.1 μm at day 29 for yogurt containing neat oil and from 3.2 μm at day 1 of storage to 3.7 μm at day 29 for yogurt containing fish-oil-in-water emulsion. For drinking yogurt without additives, Nielsen et al. (2007) found that droplets had surface area mean diameters (D[3,2]) between 1.5 and 1.7 μm.

Color has also been measured. Strawberry yogurt containing microencapsulated salmon oil tended to be slightly darker (lower L* values) and slightly more blue (higher b* values) compared to
strawberry yogurt without salmon oil, but these differences were not noticeable to untrained panelists in the Estrada et al. (2011) study. Tamjidi et al. (2012) reported that yogurt containing microencapsulated fish oil was slightly darker (lower L* value) at day 1 of storage, usually less green (higher a* value), and sometimes more yellow (higher b* value) compared to the control yogurt. The change in color of the yogurt containing the encapsulated fish oil compared to their control yogurt was relatively small (between 2 and 3), meaning undetectable by sight, and this difference was primarily due to the brownish yellow color of the gelatin used to produce fish oil microcapsules.

7.9 HEALTH BENEFITS OF OMEGA-3 FATTY ACIDS IN YOGURT

Much research has been performed on the bioavailability of fish oil. Schram et al. (2007) studied the effect of the food matrix on the bioavailability of omega-3 fatty acids, including EPA and DHA, by measuring changes in the fatty acid composition of lipids from chylomicrons in the plasma from healthy male participants over a 6-h period. They found that a yogurt drink containing added fish oil was a good matrix for supplying omega-3 fatty acids as it leads to an absorption peak for both EPA and DHA within 2 h of consumption before returning to background levels at 6 h after consumption. This rapid absorption was explained by the preformed emulsions in the yogurt (Schram et al., 2007). Sanguansri et al. (2013) studied the intestinal absorption of EPA and DHA from yogurt and other vehicles fortified with microencapsulated tuna oil powder in people with an ileostomy. It was found that 0.79% of the EPA and 0.54% of the DHA from the corresponding delivery dosage was recovered from ileal effluent over 18 h after consumption of the fortified yogurt, indicating efficient digestion and absorption of microencapsulated tuna oil powder in the small intestine. They also reported that the food matrix affects the transit time of both EPA and DHA through the gastrointestinal tract, specifically fortified yogurt and cereal bar as having a slower transit than fortified orange juice and fish oil capsules (Sanguansri et al., 2013).

The effect of fish oil on blood lipids has also been investigated. Risé et al. (2011) fed subjects yogurt enriched with 0.25% fish oil providing 184 mg/day of total omega-3 fatty acids that included 64 mg of EPA and 74 mg of DHA. The weight percentages of lipids in the blood increased from 0.40% at the start of treatment to 0.54% after 6 weeks for EPA and from 1.76% at the start to 1.95% after 6 weeks for DHA. Hlavatý et al. (2008) fed obese women a low-calorie diet containing yogurt either with or without 790 mg/day of added fish oil. They found that an increasing proportion of omega-3 fatty acids incorporated into serum lipids and undesirable changes in serum fatty acid composition were prevented with the consumption of a low-calorie diet containing added fish oil. McCowen et al. (2010) fortified yogurt with a stable emulsion of DHA from algal oil and measured plasma lipid contents in volunteers who consumed one serving of this yogurt containing 600 mg DHA daily. They reported a 32% increase in plasma phospholipid DHA content and a 7% decrease in phospholipid arachidonic acid content in the volunteers.

7.10 CONSUMER ACCEPTANCE AND SALES

The effect of fish oil fortification on consumer acceptance and sales has been mixed. Bonanno (2016) stated that fortification of yogurt with omega-3 fatty acids leads to lower consumer
acceptance and prices due to the perception of omega-3 fatty acids as not being suitable for yogurt and to off-flavors. However, Chase et al. (2009) reported that the percentage growth in sales of omega-3 fortified products, including yogurt, was greater than the percentage growth in sales of the conventional food in Canada.

### 7.11 SUMMARY

Fish oil containing omega-3 fatty acids has often been successfully incorporated into yogurt. Consumption of such fortified yogurt has been shown to increase omega-3 fatty acids in the bloodstream. However, concerns about possible lack of consumer acceptance of yogurt fortified with fish oil need to be addressed to increase sales of yogurt containing fish oil and to provide one way to help raise omega-3 fatty acid content in the bloodstream of the general population.

### REFERENCES


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8.1 INTRODUCTION

Cardiovascular diseases are the leading cause of death worldwide, and dyslipidemia, in particular elevated low-density lipoprotein (LDL) cholesterol concentrations in blood, is a major diet-related risk factor for cardiovascular diseases that can be prevented by addressing lifestyle behaviors including diet (WHO, 2009). The National Cholesterol Education Program Adult Treatment Panel III report (NCEP, 2002) recommends modification of diets, including the enrichment of diet with 2 g/day plant sterols or stanols, to improve blood lipid profile. Similarly, the European Atherosclerosis Society Consensus Panel suggested that functional foods with added plant sterols or stanols may be considered in individuals with cardiovascular risk as part of a dietary prevention strategy (Gylling et al., 2014). Plant sterols and stanols have been granted a health claim regarding their beneficial effects in the prevention of cardiovascular diseases, and functional foods with these agents such as yogurt are sold in many countries (St-Onge and Jones, 2003).

Yogurt is a comparatively new functional food for delivering plant sterols and stanols. The Codex Alimentarius Commission defines yogurt as a fermented milk product specifically characterized by the presence of the symbiotic starter cultures of Streptococcus thermophilus and Lactobacillus delbrueckii ssp. bulgaricus (Codex Alimentarius, 2011). Yogurt is accepted as an indispensable component of healthy diet in many countries, as its consumption has been associated with health and well-being for hundreds of years. The unique properties of yogurt that make it valuable are briefly listed as (1) nutrient-dense food composition—providing substantial amount of macronutrients (lactose, milk proteins, and a wide range of fatty acids) and micronutrients (calcium, potassium, zinc, phosphorus, magnesium, vitamin A, riboflavin, vitamin B₁₂), (2) high-quality protein and specific amino acids content, (3) modulator effect on gut microbiota, (4) easy to digest, (5) high bioavailability, and (6) satiating effect (Marette and Picard-Deland, 2014). Research has depicted the potential protective role of regular yogurt consumption against the nutrition-related health problems such as obesity, type 2 diabetes, cardiovascular diseases, and gastrointestinal system diseases and certain cancers as well as bone and dental health (Gijsbers et al., 2016; Glanville et al., 2015; Moreno et al., 2015; Tapsell, 2015; Astrup, 2014). As yogurt is already a unique food for a healthy and balanced diet, incorporation of plant sterols and stanols into yogurt gives the new functional food an edge over the other alternatives.

This chapter focuses on the plant sterols- and stanols-enriched yogurt, within the scope of the identification, structure, sources, metabolism, health effects, and safety of plant sterols and stanols.
8.2 Identification of Plant Sterols and Stanols

Plant sterols and stanols, also called phytosterols and phytostanols, are nonnutritive compounds that are botanically analogous to cholesterol. While cholesterol is the sterol of mammalian cells, plant sterols and stanols are typical constituents of plants’ cell walls that provide membrane fluidity and serve as hormone precursors (Brufau et al., 2008; Demonty et al., 2009).

More than 200 different plant sterol and stanol molecules have been identified in nature, however, a limited number of compounds are commonly found in the human diet; these are \( \beta \)-sitosterol, campesterol, stigmasterol, avenasterol, brasikasterol, and ergosterol. Among them, sitosterol, campesterol, and stigmasterol contribute to about 98% of the total dietary plant sterol intake. The most common stanols are \( \beta \)-sitostanol and campestanol (Moreau et al., 2002; Brufau et al., 2008).

8.3 Structure of Plant Sterols and Stanols

Like cholesterol, plant sterols and stanols belong to the family of triterpenes, with a tetracyclic ring and a side chain linked to carbon 17. The chemical structures of plant sterols and stanols differ from cholesterol only with the presence of either an extra methyl or ethyl group, or another double bond in the side chain. These compounds are classified into sterols and stanols, according to the presence or absence of a double bond at the \( \Delta 5 \) position; the stanols are saturated forms of sterols. Plant stanols are also derived from plant sterols using catalytic hydrogenation by food industry (Ellegard et al., 2007; Kidambi and Patel, 2008; Moreau, 2015). The structures of major plant sterols and stanols compared to the structure of cholesterol are given in Fig. 8.1.

8.4 Sources of Plant Sterols and Stanols

Plant sterols and stanols naturally occur in foods of plant-origin such as vegetable oils; nuts; seeds; grains and grain-derived products; vegetables including sprouts, cabbages, and cauliflowers; and fruits including olives, avocados, apples, and berries. Among these sources, the content of plant sterols and stanols largely vary depending on the genetics of the plant, geographical location, agricultural characteristics, and processing conditions. Tall oil, a product of the pulping of pinewood and vegetable oil deodorizer distillate, is the major source of plant sterols and stanols used for incorporation into commercial products (Phillips et al., 2002; Ellegard et al., 2007; Kamal-Eldin and Moazzami, 2009; Cusack et al., 2013).

Dietary intake of plant sterols and stanols varies throughout the world, depending on the consumption of plant foods. Normally, a typical Western diet includes between 150 and 400 mg of plant sterols (Ling and Jones, 1995; Katan et al., 2003; Valsta et al., 2004; Moreau, 2015). According to the European Prospective Investigation into Cancer study, plant sterol intake ranged from 178 to 463 mg/day, whereas the intake of plant stanols was about only 50 mg/day in European populations (Andersson et al., 2004). The database showed that the mean plant sterol intake was 300 mg/day for men and 293 mg/day for women, mainly provided from bread, cereals, fats, and vegetables in the UK diet (Klingberg et al., 2008). The intake was the highest among vegetarians, despite still under 1 g/day. However, these amounts of plant sterols and stanols in a normal Western diet are too small to
FIGURE 8.1

Structure of plant sterols and stanols.

have a therapeutic effect. Therefore, food industry focused on developing plant sterol- and stanol-enriched functional foods. The first functional food enriched in plant stanol fatty acid esters was Benecol spread, developed by Raisio, in Finland in 1995 (Law, 2000). Since then, many other functional foods have been developed and marketed globally (Moreau, 2015).

8.5 METABOLISM OF PLANT STEROLS AND STANOLS
Similar to dietary cholesterol, dietary plant sterols and stanols are solubilized in micelles prior to digestion and intestinal absorption. Plant sterol and stanol uptakes by the brush border membrane of intestinal lumen require interaction with the micelles. Transport proteins such as Niemann-Pick C1-Like1 and ATP-binding cassette protein facilitate this interaction (Gylling et al., 2014). The absorption rates of plant sterols and stanols in humans is quite low, and it varies depending on the type of sterol and stanol, with a general range of 0.5%–2% for plant sterols and 0.04%–0.2% for plant stanols (Ostlund et al., 2002). Following the absorption, the compounds are effectively excreted into the bile by the liver. Therefore, plasma concentrations of plant sterols and stanols are low, of the order 0.3–1.0 mg/dL for plant sterols and 0.002–0.012 mg/dL for plant stanols. These levels are about 500-fold and 10,000-fold lower than those of cholesterol, respectively, for plant sterols and stanols (Gylling et al., 2014). The circulating levels increase significantly when the functional foods with added plant sterols or stanols are consumed regularly in long term (Fransen et al., 2007). A meta-analysis of 41 randomized controlled studies examined the effect of plant sterol- and stanol-enriched food consumption on the circulating levels of these compounds. It was demonstrated that consumption of plant sterol- and stanol-enriched foods with an average dose of 1.6 g/day increases circulating sitosterol and campesterol concentrations by 31% and 37%, respectively; however, total plant sterol and stanol remain below 1% of total sterols circulating in the blood (Ras et al., 2013). The increase in circulating levels of plant sterols and stanols might be a problem especially for people with phytosterolemia, a rare inheritable autosomal recessive disease in which plasma plant sterol or stanol is elevated 50–100 fold. Although limited data is available for tissue levels of plant sterols and stanols in persons who consume plant sterol/stanol-rich foods, it is accepted that similar proportions relative to cholesterol for plant sterol and stanol are taken up into the tissues (Gylling et al., 2014).

8.6 HEALTH EFFECTS OF PLANT STEROLS AND STANOLS
Cholesterol-lowering activities of plant sterols and stanols mainly focus on the last 60 years of research. Other biological activities such as anti-inflammatory, anticarcinogenic, and antidiabetic have been proposed, however, data about these activities is very limited (Brufau et al., 2008).

8.6.1 CHOLESTEROL-LOWERING EFFECTS OF PLANT STEROLS AND STANOLS
8.6.1.1 Effects of Plant Sterols and Stanols on Total and LDL Cholesterol
Due to their structural similarity to cholesterol, plant sterols and stanols were initially studied for their ability to inhibit cholesterol absorption; and their hypcholesterolemic effects have been known since the early 1950s (Jones and Raeini-Sarjaz, 2001; Marangoni and Poli, 2010). Early studies
report that plant sterol supplementation, mainly beta-sitosterols, decreases serum cholesterol levels in patients with atherosclerotic heart disease (Pollak, 1953; Best et al., 1954; Barber and Grant, 1955; Farquhar et al., 1956). Plant sterols and stanols were used as capsules, syrups, or suspensions to achieve substantial hypocholesterolemic effects in the 1950s. Since they had poor water solubility and bioavailability, it was difficult to use them as pharmaceutical agents, and they were consequently abandoned. In the late 1990s, parallel to the development of the functional food concept, new interest in plant sterols and stanols arose, especially when the esterification of these compounds facilitated their inclusion into some food products (Brufau et al., 2008). Miettinen et al. (1995) showed that margarine with added sitostanol ester lowered total cholesterol and LDL cholesterol levels in mildly hypercholesterolemic patients. This was the first study of plant sterols or stanols that were administered through fortified foods. On the other hand, the latest technology used in the development of pharmaceutical agents enables plant sterols or stanols in the form of capsules or tablets to be more effectively compared to the earlier version of the supplements. A recent meta-analysis compared the relative efficacy of plant sterols or stanols as supplements in the form of tablets or capsules with the form of functional foods and demonstrated that both have similar cholesterol-lowering potency (Shaghaghi et al., 2013).

Randomized, double-blind, parallel, or crossover placebo-controlled studies consistently show that daily intake of plant sterols or plant stanols is associated with reductions in the levels of plasma LDL and total cholesterol (Law, 2000; Katan et al., 2003; Homma et al., 2003; Thompson and Grundy, 2005; Malinowski and Gehret, 2010; Hernandez-Mijares et al., 2010; Gupta et al., 2011). Several meta-analyses also demonstrated consistent support for the total and LDL cholesterol-lowering effects of foods with added plant sterols and stanols. Daily intake of 2 g plant sterols and stanols in foods lowers plasma total cholesterol levels by 5%–10%, and plasma LDL cholesterol levels by 6%–15%, with an average of 10%, following a 4-week supplementation protocol (AbuMweis et al., 2008; Demonty et al., 2009; Wu et al., 2009; Musa-Veloso et al., 2011; Yue et al., 2015). This reduction in plasma LDL cholesterol levels is clinically important for individuals with high-cholesterol levels and cardiovascular risk, patients on pharmacological lipid-lowering therapy, and also adults and children older than 6 years with familial hypercholesterolemia (Cofan and Ros, 2015). An additional and dose-dependent cholesterol-lowering effect of higher plant sterol or stanol intake (up to 9 g/day) has also been demonstrated by Mensink et al. (2010) and supported by following studies by Musa-Veloso et al. (2011) and Ras et al. (2014). Further investigations are required to confirm this effect, also with a consideration of potential side effects of the agents. The underlying mechanism for cholesterol-lowering effects of plant sterols and stanols has not been completely understood. However, the total and LDL cholesterol-lowering effects of these agents generally have been explained by the competitive inhibition of cholesterol absorption from the small intestine (Gylling et al., 1997; Ostlund et al., 1999; Jones et al., 2000). Plant sterols and stanols compete with intestinal cholesterol for replacing into mixed intestinal micelles, subsequently less cholesterol is incorporated into micelles, and as a result, less cholesterol is absorbed. A second reason for the underlying mechanism is transcriptional induction of genes implicated in cholesterol metabolism in enterocytes as well as hepatocytes. Plant sterol or stanol transporters localized in the membranes of enterocytes, and increased plant stanol concentrations within the enterocyte also activate cholesterol efflux through the ATP-binding cassette A1 system back into the intestinal lumen. A recent potential reason for mechanism was based on the existence of a direct cholesterol secretion from the circulation into the intestinal lumen (Plat and Mensink, 2002a,b; Patel, 2008; De Smet et al., 2012). It was demonstrated that 2 g/day plant sterols or stanols intake reduces cholesterol absorption by 30%–40% (Katan et al., 2003).
Yogurt has been used as a carrier for plant sterols and stanols since the early 2000s. Research in which yogurt was used as a food matrix for added plant sterols and stanols provided a statistically and clinically significant decrease in plasma total and LDL cholesterol levels. Daily intake of 1.0–4.0 g plant sterols or stanols, with a median of 2 g, lowered total cholesterol by 3.8%–11.2% and LDL cholesterol by 5%–15.6% in hypercholesterolemic individuals. A list of studies that investigated the cholesterol-lowering effects of yogurt with added plant sterols or stanols is given in Table 8.1.

8.6.1.2 Effects of Plant Sterols and Stanols on Other Parameters of Lipid Profile
Although some data also suggested that plant sterols and stanols may have beneficial effects on triglyceride or high-density lipoprotein (HDL) cholesterol levels (Derdemezis et al., 2010; Plat and Mensink, 2005, 2009), the evidence for HDL cholesterol- or triglyceride-lowering effects of plant sterols and stanols is still inconsistent. Indeed, it was suggested that the effect of plant sterols and stanols on plasma triglyceride levels depends on the primary lipid profile of the individual; lowering effect was recorded in subjects with borderline hypercholesterolemia, while no effect was obtained in subjects with normal blood lipid profile (Wu et al., 2009; Demonty et al., 2013). Studies using plant sterol- and stanol-enriched yogurt also obtained inconsistent results, predominantly no significant changes either in triglyceride or HDL cholesterol levels. Apart from triglyceride and HDL cholesterol, an improvement of LDL receptor affinity and a reduction in CD36 expression by the consumption of 1.6 g plant sterol ester–enriched yogurt was demonstrated, reflecting an important antiatherogenic effect (Ruiu et al., 2009).

8.6.1.3 Use of Plant Sterols and Stanols With Statins
Plant sterols and stanols favorably affect total and LDL cholesterol levels, also when coadministered with lipid-lowering drugs such as statins (Katan et al., 2003; Goldberg et al., 2006). There are studies revealing that plant sterol- or stanol-enriched yogurt can be used to deliver plant sterols or stanols in combination with cholesterol-lowering over-the-counter statins. Plat et al. (2009) compared the non-HDL cholesterol-lowering potency of plant stanol ester–enriched yogurt, statin treatment, and their combination in patients with metabolic syndrome. Daily consumption of a plant stanol ester (2 g)–enriched yogurt drink, a low-dose (10 mg) simvastatin, or the combination, respectively, lowered non-HDL cholesterol by 12.8%, 30.7%, and 35.4%. It seems that consumption of yogurt with added plant stanol esters alone or coadministered with over-the-counter statins has beneficial effects on lipid profile of patents with metabolic syndrome, confirming the convenience of yogurt as a carrier for plant sterols and stanols.

8.6.1.4 Variability in Responses to Plant Sterols and Stanols
Studies on the cholesterol-lowering efficacy of plant sterol- and stanol-enriched food products demonstrated a wide variety in the total and LDL cholesterol responses. This variation can be accounted for by the use of plant stanols or sterols, the dose of stanols/sterols, plant sterols/stanols-enriched food matrix, the form of enriched food, structure of stanol/sterols (i.e., free or ester), source and origin of sterols/stanols, the ingestion of the stanols/sterols with or without a meal, the frequency consumption of products with added stanols/sterols, the duration of treatment, the time of year when the study was conducted, the background diet, the baseline total and LDL-cholesterol levels, the cholesterol-lowering medication and combination therapy with other nutraceuticals, and the genotype of the subjects (AbuMweis et al., 2008; Clifton et al., 2004; Sanchez-Muniz et al., 2009; Gupta et al., 2011; Derdemezis et al., 2010; Cusack et al., 2013).
<table>
<thead>
<tr>
<th>References</th>
<th>Study Design</th>
<th>Times/Day</th>
<th>Meal</th>
<th>Period</th>
<th>Dose (g/day)</th>
<th>Plant Sterol or Stanol</th>
<th>Type of Plant Sterol or Stanol</th>
<th>Source of Plant Sterol or Stanol</th>
<th>Reported Change in LDL Cholesterol</th>
<th>Reported Change in Total Cholesterol</th>
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<td>Volpe et al. (2001)</td>
<td>Randomized double-blind crossover trial</td>
<td>Once</td>
<td>Anytime</td>
<td>4 × 2 weeks</td>
<td>1–2</td>
<td>Sterol</td>
<td>Free</td>
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<td>−6.7%−11.2%</td>
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<td>Mensink et al. (2002)</td>
<td>Double-blind, placebo-controlled, parallel design</td>
<td>Three times</td>
<td>Each meal</td>
<td>4 weeks</td>
<td>3</td>
<td>Stanol</td>
<td>Ester</td>
<td>Rapeseed oil</td>
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<td>Hyun et al. (2005)</td>
<td>Randomized, double-blind, and placebo-controlled study</td>
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<td>Breakfast</td>
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<td>3.3</td>
<td>Stanol</td>
<td>Ester</td>
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<td>Anytime</td>
<td>3 × 3 weeks</td>
<td>1.8</td>
<td>Sterol and stanol</td>
<td>Ester</td>
<td>Vegetable oil + sunflower oil</td>
<td>−8%−10%</td>
<td>−6%−8%</td>
</tr>
<tr>
<td>Algorta-Pineda et al. (2005)</td>
<td>Double-blind, randomized, placebo-controlled, parallel study</td>
<td>Once</td>
<td>Anytime</td>
<td>3 weeks</td>
<td>2</td>
<td>Stanol</td>
<td>Ester</td>
<td>Not given</td>
<td>−10.8%</td>
<td>−6.8%</td>
</tr>
<tr>
<td>Doornbos et al. (2006)</td>
<td>Double-blind, randomized, placebo-controlled, parallel study</td>
<td>Once</td>
<td>With a meal Without a meal</td>
<td>4 weeks</td>
<td>3</td>
<td>Sterol</td>
<td>Ester</td>
<td>Tall oil + sunflower oil</td>
<td>−9.4%</td>
<td>−7.0%</td>
</tr>
<tr>
<td>Korpela et al. (2006)</td>
<td>Parallel, double-blind</td>
<td>Once</td>
<td>Anytime</td>
<td>6 weeks</td>
<td>1.7</td>
<td>Sterol/stanol mixture</td>
<td>Free</td>
<td>Pine tree</td>
<td>−8.7%</td>
<td>−4.0%</td>
</tr>
<tr>
<td>Seppo et al. (2007)</td>
<td>Randomized, placebo-controlled, double-blind parallel</td>
<td>Once</td>
<td>Any meal or Lunch</td>
<td>5 weeks</td>
<td>2</td>
<td>Stanol</td>
<td>Ester</td>
<td>Rapeseed oil</td>
<td>−4.9%</td>
<td>−3.8%</td>
</tr>
<tr>
<td>Hansel et al. (2007)</td>
<td>Double-blind, multicenter, parallel, randomized study</td>
<td>Twice</td>
<td>Any meal</td>
<td>6 weeks</td>
<td>1.6</td>
<td>Sterol</td>
<td>Ester</td>
<td>Tall oil</td>
<td>−7.8%</td>
<td>−4.7%</td>
</tr>
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</table>

Continued
Table 8.1 Characteristics of Studies in Which Yogurt With Added Plant Sterols or Stanols Was Used for the Treatment of Hypercholesterolemia—cont’d

<table>
<thead>
<tr>
<th>References</th>
<th>Study Design</th>
<th>Times/Day</th>
<th>Meal</th>
<th>Period</th>
<th>Dose (g/day)</th>
<th>Plant Sterol or Stanol</th>
<th>Type of Plant Sterol or Stanol</th>
<th>Source of Plant Sterol or Stanol</th>
<th>Reported Change in LDL Cholesterol</th>
<th>Reported Change in Total Cholesterol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plana et al. (2008)</td>
<td>Multicenter, randomized, double-blind, placebo-controlled, parallel clinical trial</td>
<td>Once</td>
<td>Any meal</td>
<td>42 days</td>
<td>1.6</td>
<td>Sterol</td>
<td>Free</td>
<td>Not given</td>
<td>−10.6%</td>
<td>−7.9%</td>
</tr>
<tr>
<td>Niittynen et al. (2008)</td>
<td>Double-blind, crossover trial</td>
<td>Once</td>
<td>NA</td>
<td>4–8 weeks</td>
<td>1–2</td>
<td>Sterol</td>
<td>Free</td>
<td>Not given</td>
<td>−5.8%</td>
<td>−5.2%</td>
</tr>
<tr>
<td>Rudkowska et al. (2008)</td>
<td>Single-blind, randomized crossover, clinical trial</td>
<td>Once</td>
<td>Meal Snack</td>
<td>4 weeks</td>
<td>1.6</td>
<td>Sterol</td>
<td>Free</td>
<td>Tall oil</td>
<td>−6.7%−10.4%</td>
<td>−5.0%−9.6%</td>
</tr>
<tr>
<td>Plat et al. (2009)</td>
<td>2×2 factorial design</td>
<td>Once</td>
<td>Lunch or dinner</td>
<td>9 weeks</td>
<td>2</td>
<td>Stanol</td>
<td>Ester</td>
<td>Not given</td>
<td>−12.8%</td>
<td>Not given</td>
</tr>
<tr>
<td>Vásquez-Trespalacios and Romero-Palacio (2014)</td>
<td>Double-blind crossover, placebo-controlled</td>
<td>Twice</td>
<td>Any main meal</td>
<td>4 weeks</td>
<td>4</td>
<td>Stanol</td>
<td>Ester</td>
<td>Not given</td>
<td>−10.3%</td>
<td>−7.2%</td>
</tr>
<tr>
<td>Buyuktuncer et al. (2013)</td>
<td>Randomized, double-blind, and placebo-controlled study</td>
<td>Once</td>
<td>Lunch or dinner</td>
<td>4 weeks</td>
<td>1.9</td>
<td>Stanol</td>
<td>Ester</td>
<td>Not given</td>
<td>−6.3%</td>
<td>−4.6%</td>
</tr>
<tr>
<td>Parraga-Martinez et al. (2015)</td>
<td>Randomized, double-blind, and placebo-controlled study</td>
<td>Once</td>
<td>Any main meal</td>
<td>12 months</td>
<td>2</td>
<td>Stanol</td>
<td>Ester</td>
<td>Not given</td>
<td>−11.0%</td>
<td>Not given</td>
</tr>
<tr>
<td>Salo and Kuusisto (2016)</td>
<td>Randomized, double-blind, parallel group study</td>
<td>Once</td>
<td>Lunch</td>
<td>4 weeks</td>
<td>1.6–2</td>
<td>Stanols with or without 2 g camelina oil</td>
<td>Ester</td>
<td>Not given</td>
<td>−9.4%−8.1%</td>
<td>−5.5%−3.0%</td>
</tr>
</tbody>
</table>
8.6.2 OTHER HEALTH EFFECTS OF PLANT STEROLS AND STANOLS

In addition to hypocholesterolemic effects, potential beneficial effects of plant sterols and stanols on other components of cardiovascular health such as inflammation, coagulation, platelet aggregation, oxidative stress, blood pressure, and endothelial function were also proposed. Although hypocholesterolemic effects of plant sterols and stanols have been approved in many conditions, little is known about other activities of plant sterols and stanols. Since oxidative stress is one of the components of cardiovascular disease pathophysiology, and plasma levels of oxidized lipids derived from LDL cholesterol are strong predictors of acute coronary heart disease, the potential effect of daily intake of plant sterols and stanols on the level of oxidative stress was examined. Hansel et al. (2007) showed that daily consumption of 1.6 g plant stanol ester–enriched low-fat yogurt for 6 weeks provided a significant reduction both in plasma LDL cholesterol and oxidized LDL-cholesterol levels, confirming the beneficial effects of regular consumption of yogurt with added plant sterols on lipid profile without any deleterious effects on biomarkers of oxidative stress. Potential anti-inflammatory effects of plant sterols and stanols were also associated with other health conditions besides their roles in cardiovascular diseases. Studies suggest beneficial immune modulating effects of plant sterols and stanols by decreasing the production of proinflammatory cytokines and inflammatory responses (Brull et al., 2009). A recent study by Brull et al. (2016) that assessed the efficiency of plant stanol esters on immune response in asthma patients showed that consumption of 4 g plant stanol ester–enriched soy-based yogurt for 8 weeks improved the immune function in patients with asthma. However, any consistent outcomes on these biological activities have not been demonstrated in human studies yet. Further studies are required to clarify these activities (Brufau et al., 2008; Derdemezis et al., 2010; Gylling et al., 2014; Yue et al., 2015).

Plant sterols and stanols were also suggested as potential preventative compounds toward certain cancers such as stomach, lung, ovarian, and breast. Animal and cell studies proposed that plant sterols and stanols have potential to inhibit carcinogen production and cancer-cell growth, and to promote the apoptosis of cancer cells through their cholesterol-lowering effects (Woyengo et al., 2009). However, further studies are also needed to demonstrate convincing benefit of these agents on cancer (Gylling et al., 2014).

8.7 DEVELOPING FUNCTIONAL YOGURT WITH ADDED PLANT STEROLS AND STANOLS

Understanding the factors related to the characteristics of food that impact the efficiency of plant sterols and stanols would maximize the success of these agents, enable food industry to develop more efficient functional foods, and increase the benefit for consumers. It is known that the cholesterol-lowering potency of plant sterols and stanols varies depending on the food matrix, including their fatty acid composition, usage of plant sterols or stanols, the structure of plant sterols or stanols, and form of food (Cusack et al., 2013; Clifton, 2015). Therefore, commonly used methods to optimize the effect of plant sterols or stanols on cholesterol absorption were the structural modification of these compounds and dissolving plant sterols or stanols within food fats (Ostlund, 2004).
8.7.1 Food Matrix

Dissolving plant sterols or stanols within the fatty matrix of a food was thought to be essential for cholesterol-lowering efficiency. Therefore, earlier studies testing the cholesterol-lowering effects of plant sterols and stanols used high-fat food products such as spreads, margarines, and mayonnaise due to the lipophilic nature of these compounds (Ikeda and Sugano, 1998). Nevertheless, this encouraged the consumption of high-fat foods for the promotion of cardiovascular health and total fat intake and maintaining the body weight. Hence, research focused on developing new food formulations including low-fat foods using new solubilization processes with the addition of emulsifiers, such as lecithin for dispersion of plant sterols and stanols throughout the food matrix (Ortega et al., 2006). It was shown that these agents had the potential to compete with cholesterol for the absorption if a proper formulation was used. Later on, plant sterols and stanols were added to other food matrices, including low-fat milk and yogurt, cheese, juices, beverages, bakery products, cereals, chocolate, and beef (Clifton et al., 2004; AbuMweis et al., 2008; Demonty et al., 2009; Cusack et al., 2013).

Studies comparing different food matrices showed that the matrix of yogurt is an appropriate carrier for plant sterols and stanols that provides cholesterol-lowering efficiency (Volpe et al., 2001; Mensink et al., 2002; Clifton et al., 2004; Hyun et al., 2005; Algorta-Pineda et al., 2005). A meta-analysis by AbuMweis et al. (2008) showed that low-fat yogurt drinks containing plant sterols and stanols had a similar efficacy on lowering serum cholesterol levels as of plant sterol- or stanol-enriched products with higher fat content like fat spreads and mayonnaise; and its effectiveness was greater than other foodstuffs such as fruit juices, nonfat beverages, cereals, muffins, and croissants. Further, the effect of total fat content (0.1% dairy fat versus 1.5% dairy fat) in plant stanol ester–enriched yogurt on cholesterol-lowering potency was examined by Doornbos et al. (2006), and it was shown that 3 g/day plant stanol ester delivered by a single-dose yogurt lowered total and LDL-cholesterol levels significantly regardless of the fat content of the yogurt. Recent research by Gleize et al. (2016) explained that long-chain plant sterol esters incorporated into low-fat yogurt matrix provided the greatest reduction of cholesterol micellization, confirming the role of food matrix as well as the form of plant sterols on the incorporation into mixed micelles during the absorption of cholesterol.

8.7.2 Fatty Acid Composition of Food

In addition to total food content, the effect of fatty acid composition of food is also assessed in terms of cholesterol-lowering capacity of plant sterol- or stanol-enriched functional food. Since polyunsaturated fatty acids and monounsaturated fatty acids such as linoleic acid and oleic acid found in soy oil, rapeseed oil, or olive oil already have cholesterol-lowering effects, functional foods rich in these fatty acids may have greater cholesterol-lowering potential besides the effect of plant sterols and stanols. On the other hand, saturated fatty acids or trans-fatty acids generally increase plasma LDL-cholesterol levels (NCEP, 2002). Therefore, total fat content of food should be considered as an issue when the food matrix is rich in saturated or trans-fatty acids (Gylling and Miettinen, 1999; Cohn et al., 2010; Cusack et al., 2013). Choosing low-fat alternatives would be a beneficial approach to eliminate the negative effects of these fatty acids. Therefore, low-fat yogurt was used in almost all studies in which yogurt was a carrier for plant sterols and stanol. Moreover, it was speculated that the fat composition of yogurt caused bile acid to be released and excreted in significant amounts (Al-Muqbel, 2010).
8.7.3 PLANT STEROLS VERSUS PLANT STANOLS FOR CHOLESTEROL-LOWERING EFFICIENCY

The effects of plant sterols and stanols on total cholesterol, LDL cholesterol, HDL cholesterol, or triglyceride levels were compared in previous studies. Since plant stanols are saturated counterparts of the free sterols, they were thought to be more fat soluble and less absorbable, which suggests a potential difference in the degree of cholesterol displacement in the intestinal micelles. However, meta-analyses showed that plant stanols and sterols are equally efficacious at intakes similar to those currently being recommended (Katan et al., 2003; O’Neill et al., 2005; Demonty et al., 2009; Talati et al., 2010). However, a meta-analyses conducted by Musa-Veloso et al. (2011) showed that intakes of more than 2 g/day plant stanols are associated with additional reductions in LDL cholesterol, but not the intake of plant sterols, suggesting a less-marked cholesterol-lowering effect of dietary plant sterols in longer term whereas plant stanol esters maintain their efficacy. Therefore, plant stanols are proposed as more preferable agents for the long-term management of hypercholesterolemia. However, further study is required to elucidate possible differences.

8.7.4 USE OF FREE FORMS VERSUS ESTERIFIED FORMS

The structural modification of plant sterols and stanols is another common method used to increase the cholesterol-lowering efficiency of these agents. Plant sterols and stanols exist mainly in a free form and an esterified form as cholesterol does. When incorporated as functional foods, the esterified form is generally preferred. Esterification of plant sterols and plant stanols with long-chain fatty acids was suggested to have an advantage in cholesterol-lowering potency over free forms because esterified forms have a higher solubility, which enables the incorporation of plant sterols and stanols into different food matrixes, even in low-fat food (Salo and Wester, 2005; Cusack et al., 2013). However, a meta-analysis by Demonty et al. (2009) demonstrated that LDL-cholesterol-lowering efficiency was not significantly different between free and esterified plant sterols and stanols. This can be explained by the wide variety of plant sterol and stanol esters in foods in terms of fatty acids and phenol species (Esche et al., 2012). Esterified forms of plant sterols or stanols were generally used in studies in which yogurt was the food matrix (Mensink et al., 2002; Clifton et al., 2004; Noakes et al., 2005; Hyun et al., 2005; Algorta-Pineda et al., 2005; Doornbos et al., 2006; Seppo et al., 2007; Vásquez-Trespalacios and Romero-Palacio, 2014; Buyuktuncer et al., 2013), however, free plant sterols and stanols were also used in limited studies (Volpe et al., 2001; Korpela et al., 2006; Niittynen et al., 2008; Plana et al., 2008). Both free and esterified plant sterol- and stanol-enriched yogurts provided significant reduction in LDL cholesterol levels.

8.7.5 FORM OF YOGURT

The form of food is also considered as a factor that affects the cholesterol-lowering efficiency of plant sterols and stanols (Demonty et al., 2009; Clifton, 2015). It was proposed that solid foods with added plant sterols or stanols might have a larger LDL-cholesterol-lowering effect compared to liquid food with these agents, probably due to a longer transit time in the gastrointestinal tract. Demonty et al. (2009) compared the efficiency of these agents when incorporated in solid foods versus liquid foods. A larger LDL-cholesterol reduction (about 5.2%) was observed in solid foods compared to liquid foods.
when doses higher than 2 g/day were administered. Based on this evidence, it might be suggested that yogurt, especially spoonable alternatives, might have an advantage over plant sterol- or stanol-enriched milk, beverages, or juices due to its solid form. However, further evidence is needed to clarify this claim.

8.7.6 CONSUMPTION FREQUENCY OF YOGURT ENRICHED WITH PLANT STEROLS AND STANOLS

In earlier studies, it was thought that plant sterol- or stanol-enriched food should be consumed with meals or simultaneously with dietary fat for the competitive inhibition of cholesterol absorption. However, Plat et al. (2000) demonstrated that daily intake of 2.5 g plant stanols in margarine once per day at lunch or divided over three meals lowered LDL-cholesterol levels to a similar extent, about 10%, suggesting an intake of plant sterols and stanols with each meal was not necessary for their efficiency. An earlier study in which yogurt was used as the food matrix showed that consumption of yogurt with 1.0 g of plant sterol three times a day with breakfast, lunch, and dinner resulted in a 14% reduction in plasma LDL-cholesterol levels (Mensink et al., 2002). However, a similar cholesterol-lowering efficiency was obtained using a yogurt single-shot drink taken with only lunch (Seppo et al., 2007). Studies comparing the meals with plant sterol- and stanol-enriched foods, in terms of their efficiency, suggested that breakfast or fasting state in the morning are not the ideal meal for a single-dosage intake (AbuMweis et al., 2009; Cusack et al., 2013). Also, a single dose of plant sterol-enriched low-fat yogurt provided as a snack lowered total and LDL-cholesterol levels to a similar extent provided by the consumption of yogurt with a meal (Rudkowska et al., 2008). In conclusion, consumption of yogurts with 1.6–2.0 g of plant sterol or stanol with a meal resulted in 4.9%–13.5% reductions in LDL-cholesterol levels (Hyun et al., 2005; Doornbos et al., 2006; Seppo et al., 2007; Plana et al., 2008; Buyuktuncer et al., 2013). The intake of a single dose of functional foods with added plant sterol or stanol thought to increase the compliance of participants; however, breakfast is not emphasized as the ideal meal for a single-dosage intake. In terms of consumption as a snack, yogurt might have an advantage among the other functional foods with added plant sterols and stanols because it can be consumed independently as a snack.

8.8 SAFETY OF PLANT STEROLS AND STANOLS

Based on the present evidence, plant sterols and stanols have a favorable safety profile apart from two main concerns: (1) negative effects on fat-soluble vitamin status, and (2) long-term consumption in people with phytosterolemia. A side effect associated with the consumption of plant sterols or stanols is the suppression of plasma levels of fat-soluble vitamins, especially carotenoids or tocopherols due to their highly lipophilic structure, probably as a result of a reduction in the lipoproteins that are the vitamin carriers in plasma. Nevertheless, increasing consumption of fruits and vegetables by one extra serving per day easily compensates for the reduction in the status of these vitamins (Plat et al., 2000; Plat and Mensink, 2001; Noakes et al., 2002; Tuomilehto et al., 2009). As mentioned earlier, phytosterolemia is a rare inheritable autosomal recessive disease, and associated with mutations in genes coding for the ATP-binding cassette cotransporters G5 and G8 (ABCG5 and ABCG8) that result in truncated proteins with severe loss of function. Phytosterolemia is
characterized by a nonselective sterol hyperabsorption that results in dramatic elevation in plasma plant sterol or stanol levels (Kidambi and Patel, 2008). Some studies pointed to the possibility that elevated plasma plant sterol or stanol levels could contribute to the development of premature coronary artery diseases. Evidence in support of this hypothesis comes mainly from observations in phytosterolemia patients who hyperabsorb plant sterols and suffer premature atherosclerosis (Silbernagel et al., 2015). Therefore, the long-term consumption of foods with added plant sterols or stanols is not recommended for people with phytosterolemia (Berge et al., 2000; Lee et al., 2001; Brufau et al., 2008; Patel, 2008). Furthermore, long-term effects of plant sterol or stanol intake are also considered debatable as intervention periods were shorter than 8 weeks in almost all of the previous studies. Parraga-Martinez et al. (2015) examined the effectiveness of plant stanols for long term, and they showed that administration of plant stanols with yogurt for 12 months lowered LDL cholesterol by 11% in individuals with hypercholesterolemia. Apart from phytosterolemic cases, extensive safety evaluation studies have been conducted for plant sterols and stanols, and they are considered safe (Brufau et al., 2008; Hendriks et al., 2003; Patel, 2008). European Food Safety Authority (2012) approved that 3 g/day plant sterols/stanols lowers blood LDL cholesterol and reduces risk of coronary heart disease.

Moreover, commercial products in which plant sterols and stanols were incorporated were examined for safety aspects, and US Food and Drug Administration “generally recognized as safe” (GRAS) notifications were used for stanol esters, sterol esters from pine, sterol esters from vegetable oils, free sterols from pine, and free sterols from vegetable oils (Moreau, 2015).

### 8.9 CONCLUSION

Based on the evidence, national and international guidelines recommend functional foods with added plant sterols and stanols as part of a dietary prevention strategy for promotion of cardiovascular health, in particular, management of hypercholesterolemia. It has been accepted that daily intake of 2 g plant sterols and stanols lowers plasma LDL-cholesterol concentrations by a median of 10% following a 4-week supplementation protocol. Therefore, plant sterols and stanols are granted a health claim regarding their beneficial effects, and functional foods with these agents are sold in many countries. Yogurt is one of these food alternatives. Since yogurt already has unique properties that make it an important component of a healthy and balanced diet, addition of plant sterols and stanols into yogurt provides the development of a new functional food that increases the benefit for consumers.

### REFERENCES


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POTENTIAL APPLICATIONS OF PREBIOTICS TO YOGURT AND IMPACT ON HEALTH

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9.1 INTRODUCTION

Research and publication on selection, characterization, and application of prebiotics in human nutrition have been heavily carried out during the past two decades. The concept of prebiotic was introduced by Gibson and Roberfroid (1995). They defined a prebiotic as “a nondigestive food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health.” The concept of prebiotic has evolved somewhat over the years as our understanding of the gut microbiome structure and function has evolved. The current definition, agreed by consensus of a group of academic and industrial experts is: “A dietary prebiotic is a selectively fermented ingredient that results in specific changes, in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefit(s) upon host health.”

The common prebiotics, their composition and sources are given in Table 9.1. Inulin is a storage polymer found in some crops including banana, wheat, onions, leeks, garlic, Jerusalem artichoke, and chicory where the last two sources are used to produce it on a commercial scale (O’Bryan et al., 2013). It contains $\beta(2\rightarrow1)$ fructosyl-fructose glycosidic bonds, which gives it unique structural and physiological properties (Kip et al., 2006). These structures are resistant to hydrolysis by salivary and small intestinal enzymes expressed by humans. This means, therefore, that they can survive the digestive system and enter the colon where they are fermented by microorganisms leading to a range of health benefits (Kelly, 2008). Fructooligosaccharides (FOSs) are made of linear chains of fructose units, linked by $\beta$ ($2\rightarrow1$) bonds in which the number of fructose units ranges from 2 to 7 and they often terminate in a glucose unit. They are found naturally in plants such as onion, chicory, garlic, asparagus, banana, and artichoke (Sabater-Molina et al., 2009). Galacto-oligosaccharides (GOSs) are produced by enzymatic treatment of lactose by $\beta$-galactosidase to produce several oligomers of different chain lengths (Macfarlane et al., 2008). GOS is composed of a galactose chain attached to a single glucose molecule, which varies in chain length (2–8 monomers). It has linkages of $\beta(1\rightarrow4)$, $\beta(1\rightarrow2)$, and $\beta(1\rightarrow6)$ (Tzortzis and Vulevic, 2009; Whisner and Weaver, 2013). Lactulose is also a prebiotic at appropriate doses. It is considered as a synthetic ingredient and has been used as a laxative. This is a disaccharide (4-O-$\beta$-d-galactopyranosyl-d-fructose) and is composed of galactose and fructose, produced by the isomerization of lactose (Panesar and Kumari, 2011).

The newly emerging prebiotics may possess the same or more desirable properties than the more established prebiotics already in the market (Otieno and Ahring, 2012). This group includes isomaltooligosaccharides (IMOs), xylooligosaccharides (XOSs), soybean oligosaccharides (SOSs),...
lactitol, and lactosucrose. IMOs are glucosyl saccharides with α(1→6) and α(1→4) glycosidic linkages and they have been naturally found in various fermented foods and sugars in honey (Hu et al., 2013). In addition, XOSs are sugar oligomers of β(1→4)-linked xylopyranosyl, and they can be produced by chemical and/or enzymatic methods from a variety of xylan-containing raw materials and then refined by physicochemical treatments (Moure et al., 2006; Amaretti et al., 2013). SOS is a by-product of the production of soy protein. It contains the oligosaccharides raffinose, stachyose together with glucose, sucrose, and fructose (Kolida and Gibson, 2008). Lactitol (β-D-galactopyranosyl-(1→4)-D-sorbitol) is grouped as a synthetic sugar alcohol and used as a sugar replacement in low calorie foods (Majumder et al., 2011). Lactosucrose is a trisaccharide (O-β-D-galactopyranosyl-(1,4)-O-α-D-glucopyranosyl-(1,2)-β-D-fructofuranoside) formed from lactose and sucrose by enzymatic transglycosylation (Mu et al., 2013). Currently, this group can only be considered as candidate prebiotics as they have not been adequately (or in some cases, at all) tested in humans and no health benefits have been established for these carbohydrates. Their candidate prebiotic status is only based on their in vitro fecal fermentation properties.

### 9.2 CHALLENGES OF APPLICATION OF PREBIOTICS IN YOGURT

Selection of suitable prebiotics to be incorporated into yogurts needs to be considered carefully. The selected prebiotic should not change physicochemical and sensory properties of the yogurt. In addition, good stability of prebiotics during yogurt processing and subsequent storage conditions is essential to

<table>
<thead>
<tr>
<th>Type</th>
<th>Building Blocks and Linkage</th>
<th>Extraction Method</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inulin</td>
<td>β(2→1) Fru</td>
<td>Produced from chicory root</td>
<td>Marchessault et al. (1980)</td>
</tr>
<tr>
<td>Galacto-oligosaccharides</td>
<td>[β-D-Gal-(1→6)-]nβ-D-Gal(1→4) n-D-Glu</td>
<td>Synthesis from lactose by β-galactosidase</td>
<td>Torres et al. (2010)</td>
</tr>
<tr>
<td>Fructooligosaccharides</td>
<td>[β-D-Fru-(2→1)-D-Fru]n(2→1) α-D-Glu</td>
<td>Produced by transfructosylation from sucrose, or by hydrolysis of chicory inulin</td>
<td>Bornet (2008)</td>
</tr>
<tr>
<td>Lactulose</td>
<td>β-D-Gal-(1→4)-D-Fru</td>
<td>Extraction from isomerization of lactose</td>
<td>Seki and Saito (2012)</td>
</tr>
<tr>
<td>Xylooligosaccharides</td>
<td>[β-D-Xyl-(1→4)-]n</td>
<td>Produced from enzymic hydrolysis of xylan</td>
<td>Moure et al. (2006)</td>
</tr>
<tr>
<td>Isomaltooligosaccharides</td>
<td>[α-D-Glu-(1→6)-D-Glu]n</td>
<td>Synthesized by transgalactosylation of maltose</td>
<td>Ketabi et al. (2011)</td>
</tr>
<tr>
<td>Soybean oligosaccharides</td>
<td>[α-D-Gal-(1→6)-]nα-D-Glu(1→2)-β-D-Fru</td>
<td>Extracted from soybean whey</td>
<td>Karr-Lilienthal et al. (2005)</td>
</tr>
<tr>
<td>Lactosucrose</td>
<td>β-D-Gal-(1→4)-α-D-Glu(1→2)-β-D-Fru</td>
<td>Produced from sucrose</td>
<td>Li et al. (2015)</td>
</tr>
</tbody>
</table>

Fru, fructose; Gal, galactose; Glu, glucose; n, number of monomers; Xyl, xylose.
confer the intended beneficial effects for the host. Any structural or chemical changes in a prebiotic during processing and storage can lead to poor growth and a change in the selectivity of fermentation in the colon (Huebner et al., 2008). Yogurt manufacturing involves pasteurization and fermentation (Loveday et al., 2013). However, some prebiotics have been shown to be unstable at these processing conditions. FOS, for example, was shown to be hydrolyzed by exposure to pasteurization temperatures (Klewicki, 2007). The degree of hydrolysis of FOS increases with increasing time and temperature (for instance, 88°C for 25 min as opposed to 95°C for 30 s). However, at lower temperatures FOS was shown to be stable at low pH conditions (3.0–6.0) for 24 h (Huebner et al., 2008). For these reasons, FOS may not be a good candidate for application in yogurt. Other prebiotics or candidates may be more stable. For example, Wang et al. (2009) demonstrated high thermal stability of XOS during pasteurization at pH 2.5–4.0 and sterilization at pH 3.0–4.0. In addition, GOS was shown to be stable during pasteurization conditions at pH ranging from 2.7 to 4.1 (Klewicki, 2007).

Some physicochemical and organoleptic properties can limit use of prebiotics as an ingredient in yogurt. Inulin-like prebiotics show poor solubility in water at room temperature (Oliver et al., 2006). In addition, the taste of some prebiotics may interfere with the taste of the natural flavor of yogurt; FOS has sweet taste of around 30%–35% of sucrose (Kim et al., 2001). Inulin was shown to change the flavor of yogurt drinks compared to the control (Allgeyer et al., 2010). In another study, Guggisberg et al. (2009) observed higher creaminess values in set yogurt produced with higher inulin additions. In general, there is an increase in consumer demand for natural yogurt fermentation flavor, potentially restricting use of prebiotics (Jaworska et al., 2005; Florowska et al., 2016).

Incorporation of high concentrations of prebiotics could lead to poor quality attributes of yogurt. It has been reported that higher concentrations of polysaccharides can lead to separation of milk into two phases: a polysaccharide-enriched and casein-enriched phase (Srisuvor et al., 2013).

### 9.3 APPLICATION OF PREBIOTICS IN YOGURT

Yogurt is a fermented product that is the most popular dairy product worldwide (Oliveira et al., 2015). There is increased consumer demand for dairy products with added health benefits, such as yogurt containing prebiotics and probiotics (Allgeyer et al., 2010). In addition, they have been used in yogurt due to their technological advantages, and many studies have reported on the effect of prebiotics on physicochemical, microbiological, rheological, and sensory attributes of yogurt.

#### 9.3.1 EFFECT OF PREBIOTICS ON PHYSICOCHEMICAL PROPERTIES OF YOGURT

The addition of different prebiotics has been tested to evaluate changes in physicochemical properties of yogurt such as pH, titratable acidity, syneresis, and water-holding capacity. Srisuvor et al. (2013) did not observe any effect of addition of inulin and polydextrose on titratable acidity, pH, and syneresis of low-fat set yogurt. Similarly, inulin addition did not affect pH values of low-fat and whole milk set yogurt (Guggisberg et al., 2009). In another study, Vénica et al. (2015) showed that the addition of inulin had no effect on pH value of stirred yogurt. Coarsening of the acid-induced protein network, high level of syneresis, and increased permeability in yogurt were observed with the increasing level of inulin (Ipsen et al., 2001). Cruz et al. (2013) reported that the addition of oligofructose had no influence on pH of yogurt at refrigerated storage for 28 days.
9.3.2 EFFECT OF PREBIOTICS ON MICROBIOLOGICAL PROPERTIES OF YOGURT

Different prebiotics have been shown to improve viability of both probiotic and starter bacteria in yogurt (Table 9.2). The improved viability of these microorganisms in prebiotic-containing yogurt may be due to protection provided by prebiotics. Prebiotics are reported to protect microbial cells by providing extra solids (Capela et al., 2006). Furthermore, prebiotics could improve metabolic activities of probiotics, mainly of bifidobacteria and lactobacilli in the dairy products (Ranadheera et al., 2010). The addition of FOS was shown to improve the viability of Bifidobacterium animalis and B. longum in yogurt at 4°C for 28 days (Akalin et al., 2004). In another study, Capela et al. (2006) showed that mixing of FOS and inulin with yogurt mix improved the viability of Lactobacillus acidophilus, Lactobacillus rhamnosus, and Bifidobacterium spp., Bifidobacterium animalis and Bifidobacterium longum during 4 weeks of storage at 4°C. However, Cruz et al. (2013) found that incorporation of oligofructose had no effect on the viability of Streptococcus thermophilus or Lactobacillus delbrueckii ssp. bulgaricus during a storage period of 28 days at refrigerated storage.

9.3.3 EFFECT OF ADDITION OF PREBIOTICS ON TEXTURAL AND RHEOLOGICAL PROPERTIES OF YOGURT

Yogurt possesses specific rheological and textural properties. Aggregation of casein micelles and denatured whey proteins via hydrophobic and electrostatic bonds results in the three-dimensional texture of yogurt. Yogurt behaves as a weak gel and shows a time-dependent and shear-thinning flow behavior (Meyer et al., 2011). Evaluation of the rheological properties of yogurt is useful in product development, processing, handling, quality control, and storage (Velez-Ruiz, 2008). The addition of inulin to yogurt was observed to change the rheological and textural properties of the product. A low magnitude of yield stress value and firmness was observed with inulin-containing yogurt compared to yogurt without inulin (Paseephol et al., 2008). In another study, Guggisberg et al. (2009) observed higher yield stress and firmness in inulin-containing low-fat and whole milk yogurt compared to those of the

<table>
<thead>
<tr>
<th>Type of Yogurt</th>
<th>Prebiotic/s</th>
<th>Probiotic Bacteria</th>
<th>References</th>
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</thead>
<tbody>
<tr>
<td>Whole milk yogurt</td>
<td>Fructooligosaccharide (FOS)</td>
<td>Lactobacillus acidophilus, Lactobacillus casei, Lactobacillus rhamnosus, and Bifidobacterium spp.</td>
<td>Capela et al. (2006) and Akalin et al. (2004)</td>
</tr>
<tr>
<td></td>
<td>and inulin FOS</td>
<td>Bifidobacterium animalis and Bifidobacterium longum</td>
<td></td>
</tr>
<tr>
<td>Yogurt drink</td>
<td>Soluble corn fiber, polydextrose, and inulin</td>
<td>B. animalis and L. casei LA-5 and L. casei LA-5 and Bifidobacterium bifidum BB-02</td>
<td>Allgeyer et al. (2010)</td>
</tr>
<tr>
<td>Acidophilus-Bifidus (AB) yogurt</td>
<td>Inulin and lactulose</td>
<td>B. animalis and L. casei LC-01</td>
<td>Özer et al. (2005)</td>
</tr>
<tr>
<td>Low-fat yogurt</td>
<td>FOS</td>
<td>B. animalis and L. casei LC-01</td>
<td>Akalin et al. (2007) and Paseephol and Sherkat (2009)</td>
</tr>
<tr>
<td>Fat-free plain yogurt</td>
<td>Inulin</td>
<td>L. casei</td>
<td>Aryana and McGrew (2007)</td>
</tr>
</tbody>
</table>
control. Bozanic et al. (2001) reported that the firmness of cow’s milk and goat’s milk yogurt increased with the addition of inulin.

### 9.3.4 EFFECT OF PREBIOTICS ON CHANGES OF SENSORY ATTRIBUTES OF YOGURT

A quality yogurt is characterized by strong curd integrity, no sign of shrinkage and whey separation. In addition, the product should have pleasant odor and flavor (Srisuvor et al., 2013). Many studies reported that addition of a prebiotic changes the sensory attributes of yogurt such as aroma, taste, and mouthfeel. Allgeyer et al. (2010) showed that addition of inulin, soluble corn fiber, and polydextrose changed sensory attributes of a yogurt drink. In a separate study, inulin addition to low-fat and whole milk yogurt was shown to increase creaminess compared to the control (Guggisberg et al., 2009). Gonzalez et al. (2011) studied the effect of FOS on sensory properties of peach-flavored yogurt and concluded that FOS resulted in sour aroma in yogurt samples compared to that of the control yogurt without prebiotics. Yogurt containing inulin was shown to have a good flavor and texture (Seydim et al., 2005). Aryana and McGrew (2007) found that yogurt containing inulin had better body and texture compared to the control. In another study, low-fat yogurt containing FOS was shown to have better texture and taste over low-fat yogurt (Spiegel et al., 1994). An improvement in the creamy mouthfeel of stirred low-fat yogurt compared to the control was reported by Kip et al. (2006). However, a different finding was reported by Staffolo et al. (2004). They concluded that the sensory attributes of inulin-containing yogurt was not different from those of the control yogurt.

### 9.4 POTENTIAL HEALTH BENEFITS ASSOCIATED WITH PREBIOTIC CONSUMPTION

There are few reliable recorded studies on health effect of prebiotic-containing yogurt. Yogurt containing resistant starch was shown to increase stool short-chain fatty acids (SCFAs) including fecal acetate and butyrate of children (Aryana et al., 2015). Hussein et al. (2014) observed increase in stool SCFA content of men who consumed synbiotic yogurt containing B. animalis and inulin for three weeks.

Fermentation of prebiotics results in a change in the composition and activity of the colonic microbiome. Certain saccharolytic species, notably *Bifidobacterium*, *Lactobacillus*, and *Bacteroides* species are generally increased and the products of carbohydrate metabolism, short-chain fatty acids, predominate over those from protein metabolism that are potentially toxic.

Changes in microbiota composition may be less important than the SCFA products of metabolism. These are typically acetate, butyrate, and propionate, and they are produced in varying ratios from different carbohydrates. Receptors for SCFA, GPR41, and GPR43 are increasingly being found in a range of body tissues including the immune system (Kasubuchi et al., 2015). SCFAs are utilized in both the colonic and systemic levels. Butyrate is utilized by the colonic epithelial cells as an energy source. In addition, it is considered as a key nutrient that determines growth and activity of colonocytes. Butyrate may play a role as a factor against colonic disorders (Lupton, 2004). Furthermore, SCFAs can be absorbed in the blood stream. Acetate can be metabolized in the brain and muscles. The liver can use propionate and help to reduce production of cholesterol by modifying its synthesis (Slavin, 2013).

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children (Aryana et al., 2015). Hussein et al. (2014) observed increase in stool SCFA content of men who consumed synbiotic yogurt containing \textit{B. animalis} and inulin for 3 weeks.

The range of postulated physiological effects of prebiotic fermentation is summarized in Fig. 9.1.

### 9.4.1 REGULATION OF BOWEL HABITS

Consumption of prebiotics can have a positive impact on bowel motor function. In fact this is the only health claim yet approved by the European Food Safety Authority (EFSA). Micka et al. (2016) conducted a study in 44 healthy adults fed inulin or maltodextrin placebo at 12 g/day in a randomized double-blind placebo controlled crossover study. They found significant increases in stool frequency and in satisfaction score using a quality of life questionnaire when consuming the prebiotic relative to the placebo. Stool consistency also improved on the prebiotic.

The early literature on bowel habits has been systematically reviewed in a meta-analysis by Yurrita et al. (2014). This analysis revealed a consistent positive effect on stool frequency, stool consistency although effects on pain and abdominal distension were inconsistent.

Based on the totality of evidence now available, EFSA has approved the claim “chicory inulin contributes to maintenance of normal defecation by increasing stool frequency,” further commenting that “12 g of ‘native chicory inulin’ should be consumed daily.”
The mechanism for the effect is believed to be stimulation of peristalsis in the gut due to increased gut contents and chemical signaling due to the SCFAs produced (Micka et al., 2016).

### 9.4.2 METABOLIC HEALTH

There is abundant evidence from animal studies, mainly in mice, that prebiotic consumption can improve intestinal barrier function and reduce the levels of circulating bacterial lipopolysaccharide, a powerful inducer of inflammation (Cani et al., 2009). This has attracted a lot of attention in recent years due to the chronic inflammation and resultant impaired glucose response present in type 2 diabetes and obesity. Cani et al. (2009) used an Ob/ob mouse model to study diet and inflammation. They fed mice with either a control chow or chow supplemented with either inulin or nonfermentable cellulos. Feeding with inulin resulted in significantly higher populations of bifidobacteria, lactobacilli, and Clostridium cocoides/Eubacterium rectale cluster. Concomitant with these changes, the expression of ZO-1 and occludin, proteins involved in tight junction formation, also increased. Gut permeability, as measured by translocation of FITC-dextran across the gut, decreased significantly on administration of the prebiotic. Markers of inflammation also reduced in line with the increase in gut integrity.

Such detailed mechanistic studies are difficult to perform in humans but studies in volunteers have shown positive impacts on inflammation. Vulevic et al. (2013) fed 5.5 g of either GOS or a placebo (maltodextrin) per day for 12 weeks to 48 adult volunteers who presented at least three risk factors for metabolic syndrome. Consumption of GOS significantly increased the population of Bifidobacterium and reduced the populations of Bacteroides and Clostridium histolyticum group. Populations of Lactobacillus/Enterococcus species, Atopobium cluster, Clostridium cocoides/Eubacterium rectale group, Clostridium cluster IX, Eubacterium cylindroides, Eubacterium hallii, β-Proteobacteria, and Faecalibacterium prausnitzii were unchanged. The participants who took the prebiotic displayed reduced levels of the inflammatory marker, calprotectin, at the end of the study. They also found that total cholesterol levels were reduced after 12 weeks and, interestingly, the male volunteers displayed greater reductions than the female participants. Such gender effects are rarely reported in prebiotic studies.

Prebiotic consumption has been reported to increase feelings of satiety (Kellow et al., 2014), although there is a lack of a clear link with energy intake and very few studies had a long enough duration to show a consistent effect on weight loss. One study that has shown a potential impact of weight loss in obese individuals is that of Parnell and Reimer (2009). These authors fed 48 obese individuals 21 g of fructooligosaccharides or maltodextrin per day for 12 weeks on a randomized, placebo controlled, double-blind study. The study showed an impact on gut hormones regulating satiety and a reduction in energy intake. Volunteers on the prebiotic lost 1.03 ± 0.31 kg in weight over the 12 weeks.

Prebiotic consumption in this trial further improved the impaired glucose response characteristic of type 2 diabetes, a result seen in other studies (Kellow et al., 2014).

### 9.4.3 REDUCTION OF INCIDENCE OF INFECTIONS

Prebiotics almost invariably result in an increase in bifidobacteria, amongst other changes in the microbiota. Bifidobacteria are known to produce antimicrobial agents in addition to SCFAs, all of which can inhibit pathogenic microorganisms. Whilst this can be shown in vitro, establishing this in vivo in human studies is very difficult. Clearly direct challenge experiments are impossible and studies generally
focus on groups at risk of acquiring infections. Two such groups are travelers and those undergoing antibiotic treatment with broad-spectrum antibiotics. A study on traveler’s diarrhea was carried out by Drakoularakou et al. (2010). GOS (5.5 g) or maltodextrin placebo was fed per day to 159 volunteers who were departing on holidays to high-risk destinations around the world. Data acquisition was by use of disease score and quality-of-life questionnaires. The volunteers consuming the placebo had significantly more bowel movements and reduced stool consistency compared to the volunteers consuming GOS. The GOS also resulted in significantly reduced duration and pain in those volunteers who experienced traveler’s diarrhea.

Antibiotic-associated diarrhea is caused by an antibiotic-induced imbalance in the gut microbiota leading to overgrowth of pathogens such as Clostridium difficile. The concept of “fortifying” the gut with prebiotics to protect against this condition is, therefore, an attractive one. Lewis et al. (2005a) carried out a study feeding 12 g fructooligosaccharides or sucrose placebo per day to patients over the age of 65 who were taking antibiotics. This study, however, failed to see any protective effects of the prebiotic. Given the devastating effects of antibiotics on the gut microbiome, however, this is not perhaps too surprising. The same authors then carried out a study (Lewis et al., 2005b) in patients recovering from antibiotic-associated diarrhea. Around 10%–20% of such cases recur. They fed 142 recovering patients the same treatment regime as before and this time found that fructooligosaccharides reduced the relapse rate from 34.3% to 8.3%. This suggests that prebiotics may have a role in “rebuilding” the gut microbiome after antibiotic treatment.

9.4.4 INCREASING MINERAL UPTAKE

As already established, prebiotic consumption results in production of SCFAs in the colon. The reduction in local pH is postulated to increase solubility of calcium and facilitate its transport across the gut epithelium. Abrams et al. (2005) carried out a long-term study over 1 year on 95 male and female adolescents aged 9–13. They were fed 8 g of a mixture of fructooligosaccharides and inulin or maltodextrin placebo per day. Calcium absorption was significantly enhanced in the prebiotic group (37.7% vs. 31.7% after 1 year), and the bone mineral calcium content increased significantly more over the year than it did in the placebo group.

More recently, Whisner et al. (2013) fed either 0, 5, or 10 g GOS/day for 3-week periods. They investigated the effect on the total bacteria and bifidobacteria populations in the gut by qPCR and found that there was a concentration-dependent effect with the 5-g GOS group showing significant elevation in population of bifidobacteria from 10.89% to 22.8% of the total. The 10-g GOS group had a slight but nonsignificant increase to 11.54% of the total population. Interestingly the fractional absorption of calcium significantly increased only in the 5-g GOS/day group.

9.4.5 NONFERMENTATION-MEDIATED EFFECTS

The majority of the health benefits associated with prebiotics are believed to arise from changes in population and activity via selective fermentation. However, it is becoming apparent that certain prebiotics, mainly GOSs, have other activities that may prove to be important in the gut. Oligosaccharides are present at the cell surface of mammalian cells and appear to be involved in cell communication. Many of these contain terminal β-galactose residues, and there may be a degree of receptor mimicry with GOS.
GOSs have been recently shown to stimulate cytokine production from CACO-2 cells (Zenhom et al., 2011) and to enhance mucosal barrier function by upregulating goblet cell secretory products (Bhatia et al., 2015) and improving barrier function in CACO-2 cells (Akbari et al., 2016). It is not clear, however, to what extent such direct effects might be responsible for the observed cytokine expression and increase in barrier function on prebiotic consumption.

Another potential effect of GOS in the gut is to act as an antiadhesive to reduce the adhesion of pathogens. GOS has been shown to reduce adhesion of enteropathogenic *Escherichia coli* to HEp-2 cells in culture (Shoaf et al., 2006) in a dose-dependent manner and to inhibit cholera toxin adhesion to its receptor glycoprotein (Sinclair et al., 2009). It is essentially impossible, however, to determine if these activities occur in the human colon.

### 9.5 Conclusion

Prebiotics have been shown to have a significant health effects in humans and there is a greater possibility for incorporation of prebiotics in various types of yogurt. Although evidence to support health benefits is accumulating, we need more good-quality human studies with defined health outputs if we are to obtain regulatory approval. A range of molecular microbiological and metabonomic techniques should be used in these studies to obtain a systems-level mechanistic understanding of the mechanisms involved.

### REFERENCES


10.1 NONDAIRY BEVERAGES, THE FUTURE OF FUNCTIONAL FOODS

Over the last decade, the demand for “healthy” foods and beverages has increased on a global scale, and the diffusion of functional foods throughout the market overlapped the interests of the pharmaceutical and food sectors (Corbo et al., 2014). Nowadays, the advances in scientific research support the idea that diet may fulfill nutritional needs and exert a beneficial role in some diseases (Otles and Cagindi, 2012). Several critical factors have been recognized as the key factors leading to the diffusion of functional foods: health deterioration, due to busy lifestyles, consumption of convenience foods, and insufficient exercise; increased incidence of self-medication; increased awareness of the link between diet and health due to information by health authorities and media on nutrition; and a crowded and competitive food market (Granato et al., 2010). Above all, the various stakeholders have perceived the economic potential of functional food products as an important part of public health prevention strategies. According to the recent literature and legislation, functional foods can be defined as “foods and food components that provide a health benefit beyond basic nutrition” (Serafini et al., 2012), and in particular as a “food similar in appearance to, or may be, a conventional food that is consumed as part of a usual diet, and is demonstrated to have physiological benefits and/or reduce the risk of chronic disease beyond basic nutritional functions” (Lau et al., 2013). According to these definitions, foods in which a component has been modified by enzymatic, chemical, or biotechnological means to provide a benefit are included (Pravst, 2012).

Among the large number of novel and innovative functional foods under investigation or already present in the market, beverages are considered the most promising category because of (1) convenience and possibility to meet consumer demands for container contents, size, shape, and appearance; (2) ease of distribution and better storage for refrigerated and shelf-stable products; and (3) great opportunity to incorporate desirable nutrients and bioactive compounds (Corbo et al., 2014). Several different types of commercial functional beverages are today available (e.g., dairy-based beverages including probiotics and minerals/ω-3-enriched drinks, sports and energy drinks), and there is a growing interest toward nondairy beverages, made with vegetables, fruits, and cereals (Corbo et al., 2014; Granato et al., 2010; Kandylis et al., 2016).

Milk has been long considered as the only food containing all the essential substances for human nutrition. However, a study has recently reported that some milk constituents and common contaminants (such as pesticides, estrogen, and insulin-like growth factor 1) might be responsible for adverse reactions on the consumer’s health (Davoodi et al., 2013). Moreover, lactose intolerance/lactose malabsorption and cholesterol content are major drawbacks related to functional dairy products (Prado et al., 2008). Up to 70% of the world population has lactase nonpersistance, although lactose tolerance
could be affected by many nutritional and genetic factors. In Europe, the prevalence of lactose intolerance is about 5% in the British population and has increased to 17% in Finland and northern France (Lomer et al., 2008). The prevalence is above 50% in South America, Africa, and Asia, reaching almost 100% in some Asian countries (Zannini et al., 2013). In the United States, the prevalence is 15% among whites, 53% in Mexican Americans, and 80% in the African American population. Australia and New Zealand have a prevalence of lactose intolerance of 6%–9% (Tomar, 2014).

The health issues, in combination with the growing trend of vegetarianism and the limited use of dairy products in the diet of many countries, especially in Asia and Africa, has led to the development of nondairy beverages, designed as functional products also suitable for the delivery of probiotics (Granato et al., 2010). Furthermore, nondairy beverages are also considered as a more economical alternative to dairy products in developing countries. Nondairy beverages in the form of traditional products have long existed all over the world (such as boza, bushera, chhang, chica, haria, mahewu, omegisool, pozol, togwa) mainly based on cereals (Kandylis et al., 2016). In addition to these, several new nondairy probiotic beverages have been developed (Soccol et al., 2012). A huge potential has been recognized to using cereals as vehicles for probiotics and functional compounds such as antioxidants, dietary fiber, minerals, prebiotics, and vitamins (Nionelli et al., 2014); this is the reason why different commercial cereal-based beverages are today available on the market, such as Proviva (Skane Dairy, Sweden), the first oat-based probiotic food beverage produced using Lactobacillus plantarum 299v (Prado et al., 2008), and Whole Grain Probiotic Liquid (Grainfields, Australia), a refreshing, effervescent beverage containing both lactic acid bacteria (LAB) (Lactobacillus acidophilus, Lactobacillus delbrueckii) and yeasts (Saccharomyces cerevisiae var. boulardii and S. cerevisiae) as well as vitamins, amino acids, and enzymes (Soccol et al., 2012) (Table 10.1).

### 10.2 LACTIC ACID BACTERIA AS STARTERS FOR CEREAL-BASED BEVERAGES FERMENTATION

#### 10.2.1 ADVANTAGES OF FERMENTATION

Fermentation has long been used as a way to naturally enhance the food matrix, without the need for additives or preservatives (Hugenholtz, 2013). In order to facilitate the control and reproducibility of final product qualities, the industry uses defined starter cultures. LAB have long been used as such in many food substrates, e.g., milk, meat, vegetables, and cereals, as well as being part of their indigenous microflora (Holzapfel, 1997), and many of them have been granted with the “generally recognized as safe” (GRAS) status. The genera Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Pediococcus, Streptococcus, and Weissella are naturally found on the surface of grains and in the surrounding environment (Guyot, 2012). For this reason they often form the natural inoculum, together with fungi, of fermented cereal gruels commonly consumed in many rural societies worldwide (Nout, 2009). Fermentation of cereal-based beverages by LAB has been shown: (1) to improve protein digestibility (Holzapfel, 1997; Peyer et al., 2016), due to the proteolysis occurring during fermentation (Coda et al., 2014; Nionelli et al., 2014); (2) increase nutritional bioavailability of minerals through the degradation of phytic acid by the activation of endogenous phytases and/or due to the contribution of microbial phytases (Coda et al., 2014; Nionelli et al., 2014); (3) increase the bioaccessibility of other nutrients (e.g., polyphenols, and fibers) (Coda et al., 2015; Nionelli et al., 2014); (4) decrease the glycemic index through the biological acidification and affect the rate of
### Table 10.1 Traditional, Commercial, and Experimental Cereal-Based Fermented Beverages

<table>
<thead>
<tr>
<th>Name</th>
<th>Cereal</th>
<th>Microorganism</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Traditional</td>
<td></td>
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<tr>
<td>Boza</td>
<td>Barley, oats, rye, millet, maize, wheat, rice</td>
<td>Lactic acid bacteria (LAB) and yeasts</td>
<td>Akpinar-Bayizit et al. (2010)</td>
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<td>Bushera</td>
<td>Sorghum</td>
<td>LAB</td>
<td>Muyanja et al. (2003)</td>
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<td>Chhang</td>
<td>Millet</td>
<td>LAB and yeasts</td>
<td>Thakur et al. (2015)</td>
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<td>Chicha</td>
<td>Rice</td>
<td>LAB and Bacillus spp.</td>
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<td>Millet</td>
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<td>Kandylis et al. (2016)</td>
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<td>Pozol</td>
<td>Maize</td>
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<td>Oat, wheat, barley</td>
<td>L. plantarum</td>
<td>Salmerón et al. (2009)</td>
</tr>
<tr>
<td></td>
<td>Sorghum</td>
<td>–</td>
<td>Muyanja et al. (2012)</td>
</tr>
<tr>
<td></td>
<td>Oat</td>
<td>Bifidobacterium spp., LAB</td>
<td>O’Connor et al. (2005)</td>
</tr>
<tr>
<td></td>
<td>Oat bran</td>
<td>Lactobacillus rhamnosus</td>
<td>Loponen et al. (2007)</td>
</tr>
<tr>
<td></td>
<td>Whole-grain</td>
<td>L. plantarum</td>
<td>Loponen et al. (2007)</td>
</tr>
<tr>
<td>Oagurt</td>
<td>Oat</td>
<td>L. acidophilus, Lactobacillus casei, Bifidobacterium</td>
<td>Walsh et al. (2010)</td>
</tr>
<tr>
<td></td>
<td>Oat</td>
<td>L. plantarum</td>
<td>Nionelli et al. (2014)</td>
</tr>
<tr>
<td></td>
<td>Oat</td>
<td>Lactobacillus reuteri, L. acidophilus, Bifidobacterium bifidum</td>
<td>Mårtensson et al. (2001)</td>
</tr>
</tbody>
</table>
resistant starch (De Angelis et al., 2009); (5) prolong shelf life through the acidification and the release of antimicrobial compounds (Angelov et al., 2006; Gupta et al., 2010); and (6) enhance organoleptic quality (Nionelli et al., 2014; Peyer et al., 2016). The favorable macro- and micronutrients profile of cereals has made them an excellent candidate for functional food production through LAB fermentation (Blandino et al., 2003), providing the necessary environment for their growth while at the same increasing the bioaccessibility of these compounds. Bioaccessibility, which is the release of the compound from its natural matrix to be available for intestinal absorption, is in fact the first limiting factor to bioavailability (Endo and Dicks, 2014). The bioavailability of nutrients that are usually bound as reserve molecules in the form of starch and proteins can be enhanced with the addition of malted cereals, either directly or by adding to the pool of hydrolytic enzymes with, e.g., amylases, glucanases, and peptidases (Gupta et al., 2010; Nionelli et al., 2014).

### Table 10.1 Traditional, Commercial, and Experimental Cereal-Based Fermented Beverages—cont’d

<table>
<thead>
<tr>
<th>Name</th>
<th>Cereal</th>
<th>Microorganism</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>–</td>
<td>Oat</td>
<td>L. plantarum</td>
<td>Angelov et al. (2005)</td>
</tr>
<tr>
<td>–</td>
<td>Oat</td>
<td>L. plantarum, Lactobacillus paracasei ssp. casei, L. acidophilus</td>
<td>Gokavi et al. (2005)</td>
</tr>
<tr>
<td>Commercial</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proviva</td>
<td>Oat</td>
<td>L. plantarum 299v, L. acidophilus, L. del-brueckii, Saccharomyces cerevisiae var. boulardii, S. cerevisiae</td>
<td>Prado et al. (2008)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>L. acidophilus and B. bifidum</td>
<td>Soccol et al. (2012)</td>
</tr>
<tr>
<td>Whole grain probiotic liquid</td>
<td>Oat</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yosa</td>
<td>Oat</td>
<td></td>
<td>Blandino et al. (2003)</td>
</tr>
</tbody>
</table>

All the traditional beverages are produced by spontaneous fermentation, without the addition of starters, and can be characterized by various structures (liquid to semiliquid texture), while all the experimental beverages reported in the table are characterized by a yogurt-like texture.

10.2.2 **FUNCTIONALITY**

Besides acidification, LAB also have the ability to contribute to the synthesis and the release of several important nutraceuticals (Waters et al., 2015). The mechanisms by which LAB fulfill the role of efficient cell factory for the production of functional biomolecules were largely demonstrated for cereal-based beverages (Coda et al., 2011; Nionelli et al., 2014). Cereal fermentation leads to the decrease in the carbohydrates level as well as some not-digestible poly- and oligosaccharides, while the availability of certain amino acids and B vitamins is improved (Gobbetti et al., 2010). Furthermore, other compounds having biological functions such as gamma-amino butyric acid and biogenic peptides can be produced especially when selected LAB are used (Coda et al., 2010, 2012). Indeed, the selection of appropriate starter cultures for different kinds of cereal beverage is needed by the industry to drive, accelerate, and standardize the fermentation (Coda et al., 2014; Nionelli et al., 2014).
An important step to improve the nutritional value of fermented foods is through the activity of functional bacteria (Gobbetti et al., 2010) such as probiotics. The most important traits for a promising probiotic rely upon (1) survival in low pH and with bile salts added; (2) adhesion to intestinal epithelium; (3) antimicrobial activity toward foodborne pathogens and competitive adhesion to mucosa; (4) immunomodulation; (5) safety issues (production of harmful metabolites, like biogenic amines), and (6) transmission of genes encoding antibiotic resistance (Nagpal et al., 2012; Sip and Grajek, 2009). In addition to these characteristics, many LAB and Bifidobacterium spp. have been reported to produce vitamins such as folate, cobalamin, menaquinone (vitamin K), riboflavin, and thiamine. The use of these cultures in food fermentation potentially provides routes not only to enhance the nutritional profile of the food but also to deliver microorganisms to the gut, where they can synthesize such vitamins in vivo (O’Connor et al., 2005). It is commonly thought that the microbial colonization of the intestinal epithelium can give the best effect, since they can affect the intestinal immune system, displace enteric pathogens, provide antimitagens and antioxidants, and possibly other effects by cell signaling (Park and Floch, 2007). Apart from these classical properties, some additional features are required for probiotics in functional beverages, like the interaction with the starter cultures, as antagonistic interaction between probiotics and starter cultures may result in growth inhibition by acid, peroxide, bacteriocins, and other metabolites (Brajdes and Vizireanu, 2013; Nagpal et al., 2012). Similarly, the ability of probiotics to grow well in cereal or fruit and vegetable juices could depend, respectively, on their ability to exhibit amyloytic activity or to resist the effects of preservatives (Yeo et al., 2011).

Due to the fact that the prolongation of shelf-life is a great challenge for functional beverages, some researches have tried to improve the viability of probiotics. They should be maintained in the food product until the time of consumption and be present in significant numbers, at levels of at least $10^7$ viable cells per gram or milliliter. Many approaches have been proposed, like a modification of the atmosphere of the product based on the increase of the content of CO$_2$ in the headspace, which might have an impact on the survival of microaerophilic and anaerobic bacteria (Walsh et al., 2014). Also, addition of ascorbic acid (vitamin C) might have a protective effect on probiotic cells during storage, presumably because it is an oxygen scavenger, thus promoting a more favorable anaerobic environment (Shah et al., 2010). A further aspect related to the exploitation of microorganism functionality includes the production of bacterial polysaccharides. The in situ production of oligosaccharides and exopolysaccharides (EPSs) by Weissella spp. was previously reported also for different cereals (Kajala et al., 2016; Katina et al., 2009; Zannini et al., 2013). It has been suggested that the health-promoting effects of EPS-producing strains are related to the biological activities of these biopolymers. Exopolysaccharides might contribute to human health by affecting gastrointestinal functionality as prebiotics or due to antitumor, antulcer, immunomodulating, or cholesterol-lowering activities (De Vuyst and Degeest, 1999). Some probiotic bacteria also are able to produce EPS and have been already employed for several fermented dairy products (Ruas-Madiedo et al., 2002). The ability to affect the texture by acting as emulsifiers or stabilizers is one of the most important features of EPS and is discussed later.

Finally, it should be emphasized that the health benefits imparted by probiotic microorganisms are strain specific, and that not even strains of the same species will be effective against defined health conditions or will provide all proposed benefits (Shah et al., 2010). As not all the desirable properties are expressed by all probiotic microorganisms, the screening and selection of potential probiotic starters is still important (Kumura et al., 2004; Ouwehand et al., 2002).
10.2.3 EFFECT ON TEXTURE AND SENSORY QUALITY

The ability of some LAB to excrete high-molecular-weight polysaccharides that can increase the viscosity of the liquid substrate is a very relevant feature in the production of cereal-based yogurt-like beverages. EPS are formed through polymerization of sugar subunits and can be either composed from repeating glucose or fructose subunits (homopolysaccharides) or from two or more different subunits (heteropolysaccharides) (Galle and Arendt, 2014). The type of EPS produced and its amount depends principally on the sugars present in the medium (Galle and Arendt, 2014), which can act as substrate or as acceptor molecules, on the presence of micronutrients (e.g., minerals acting as enzymes cofactors), and the environmental conditions of fermentation (e.g., incubation temperature and time) (Kajala et al., 2016).

The in situ production of EPS is of particular interest to manufacturers of fermented cereal-based drinks as they can resemble dairy products (Peyer et al., 2016). These products can therefore make a “natural,” “additive free” claim and, at the same time, avoid the costs that result from the expensive and laborious EPS purification procedures (Badel et al., 2011). Dextran, for instance, a flavorless homopolysaccharide composed of glucose subunits, is a GRAS-granted thickener already used by the food industry (Peyer et al., 2016).

The ability of LAB to release texture-enhancing EPS has been found in many starter cultures involved in the production of traditional beverages. For instance, a significant number of LAB strains isolated from Nigerian ogi (sorghum-based) and fufu (cassava-based) were EPS producers (Adebayotayo and Onilude, 2008). L. delbrueckii ssp. bulgaricus NCFB 2772 was found to enhance to a greater extent the viscosity of an oat-based medium when glucose was present as a supplementary carbon source instead of fructose, which was seen to cause the release of EPS with lower relative molecular mass (Grobben et al., 1997). Similarly, Mårtensson et al. (2002) studied the possibility of developing a yogurt-like ropy product derived entirely from oats and water by employing an EPS-producing strain of Pediococcus damnosus in combination with an ordinary yogurt starter culture. A sensory preference test successfully showed no significant difference between the flavored, nondairy product and a dairy equivalent control. Similarly, the textural properties of a beverage formulated with gelatinized emmer flour (30% w/w in tap water) and added sucrose (10% w/w) were enhanced using EPS-forming species of Weissella cibaria as inoculum. The fourfold viscosity increase, compared to a control fermented by EPS-negative L. plantarum strain, conveyed a texture similar to yogurt to the final product (Coda et al., 2011). In a recent study, the potential of two cereal-associated W. cibaria strains to produce exopolysaccharides in situ during the development of a prebiotic drink based on barley malt extract was examined (Zannini et al., 2012). The higher viscosity positively influenced the mouthfeel of the beverage. Some cereal flours, when mixed with water, can lead to a porridge-like texture because of the high content of molecules like starch and β-glucans that have a viscosity-enhancing effect (Lorri and Svanberg, 2009). This characteristic is considered important in certain African countries, where maize, sorghum, or millet porridges represent a crucial energy source as weaning food for young children (Humblot et al., 2014). In order to maintain a high-energy density in these formulations without the need for watering down, LAB with enzymatic activity have been employed for partially degrading these biopolymers (Onyango et al., 2004). Among these, amylolytic LAB (ALAB) that are able to degrade polysaccharides have been isolated from many traditional beverages (Guyot, 2012). The biodiversity of ALAB is quite limited and the most prominent ones belong to the species Lactobacillus manihotivorans, Lactobacillus fermentum, Lactobacillus amylovorus, Lactobacillus amylophilus, L. plantarum, and Lactobacillus amylyticus (Reddy et al., 2008).
Raw cereals carry very low levels of organoleptic-active compounds, and in this form, confer flat, and often unpleasant odors and flavors (Peyer et al., 2016). The bitterness and astringency carried by certain phenolic compounds found in the outer layers of whole grains can also lead to poor acceptance (Peyer et al., 2016). Together with other technological processes such as boiling, toasting, and roasting (Coda et al., 2011), fermentation has been used to improve sensorial and textural properties of cereal-based beverages (Nionelli et al., 2014). Investigations on sensorial changes in cereal matrices after LAB fermentation in cereal matrices have been initially done in relation to off-flavor formation during microbial spoilage in beer (Peyer et al., 2016). More recently, research has concentrated on the flavor and textural changes caused by the inoculation of selected LAB (Peyer et al., 2016), particularly in the case of probiotic addition (Coda et al., 2011; Salmerón et al., 2014), or for quality improvement of traditional cereal-based fermented beverages (Blandino et al., 2003) (Table 10.1). Overall, it was demonstrated that the use of defined starter cultures led to a product with better appearance, aroma, taste, and acceptability than spontaneously fermented beverages (Peyer et al., 2016). The majority of work done on novel cereal-based fermented beverages has used L. plantarum as starter culture because of its robustness under conditions of low pH (Charalampopoulos et al., 2002). This trait often gives this species a competitive advantage against other autochthonous microorganisms present on the grains, and the ability to deliver a pleasant organoleptic profile in the form of “dairy”-related flavors (e.g., diacetyl, acetoin, acetaldehyde) (Prado et al., 2008; Salmerón et al., 2015). However, a defined starter does not preclude the release of specific flavors when inoculated in different cereal matrices. The microbial flavor compounds released by L. plantarum NCIMB 8826 after fermentation of four different gruels (oat, wheat, barley, and barley malt) were present at varying concentrations depending on the cereal used (Salmerón et al., 2009). Moreover, none of the metabolites were common for all substrates, indicating a complex flavor-formation interdependency that exists between bacterial culture and substrate components (Peyer et al., 2016). Carbohydrates, amino acids, and other chemical compounds (e.g., organic acids, fatty acids) present in cereals, or released from LAB during fermentation, can be channeled into different metabolic pathways that ultimately lead to specific organoleptic compounds (Gänzle et al., 2007).

Organic acids (lactic, acetic) are the main compounds deriving from the sugar metabolism of LAB. The extent to which lactic and acetic acid accumulate depends primarily on the metabolism of the specific starter and on the substrate supply, but fermentation conditions, e.g., temperature, buffering capacity, affect culture viability (Helland et al., 2004) and determine the extent of acids released in the medium as well. For example, if compounds that can function as alternative electron acceptors are present in the medium, pyruvate can be channeled into alternative metabolic pathways (Liu, 2003), increasing the ratio of acetic to lactic acid released in the media (Kandler, 1983). Lactic acid, quantitatively the most important organic acid found after LAB fermentation, is odorless but in aqueous solution imparts a mild acidic note (Hartwig and McDaniel, 1995). The “sour” perception of lactic acid in beverages carries important thirst-quenching properties and consequently has been exploited in novel refreshing products (Warner, 2010). Acetic acid, compared to lactic acid, is released in lower concentrations, but because of its lower taste threshold and higher volatility, it can become perceptible as pungent-sour with a “cider-vinegar” aroma (Burdock, 2002). Finally, the importance of the pH after fermentation, often found between 3.0 and 4.5, as a factor influencing the final acceptance of a novel beverage should also be considered (Salmerón et al., 2015). In fact, the increase in sourness coincides with a general decrease in sweetness (McFeeters, 2004), unless the release of sugar moieties exceeds again the sugar consumption, leading to a consequent increase of the sweet taste (Mugula et al., 2003).
The contribution of LAB to flavor and taste on volatile and nonvolatile fractions of cereal-based beverages has been recently reviewed (Peyer et al., 2016). The nonvolatile fractions include primarily sugars and some carboxylic acids that contribute to the sweet and sour taste of the beverages. The volatile fraction, including compounds perceived as odor and flavor, comprises carboxylic acids, alcohols, aldehydes, ketones, and esters (Peyer et al., 2016). The main volatile compounds of cereal-based beverages fermented by LAB have been mostly associated with the carbohydrate and amino acid metabolisms (Peyer et al., 2016). Diacetyl (butane-2, 3-dione) is a ketone responsible for a butterscotch-like aroma (Burdock, 2002), mainly formed during sugar, citrate, and amino acid catabolism (Hugenholtz et al., 2000). Metabolically related to diacetyl are the less flavorsomeacetoin, formed by the reduction of diacetyl or after enzymatic decarboxylation of α-acetolactate, and 2,3-butanediol, which results from the reduction of acetoin (Axelsson, 1998). Although considered as being off-flavors in beer (Bokulich and Bamforth, 2013), these low-molecular-weight compounds are also responsible for mellowing the flavor during cereal fermentation (Mugula et al., 2003). Only some LAB species, e.g., Lactococcus lactis, L. plantarum, and Oenococcus oeni, are able to metabolize citrate to pyruvate that can be redirected into the acetoin/diacetyl pathway (Hugenholtz, 1993). High levels of diacetyl and acetoin can also be found due to alternative metabolic patterns (Peyer et al., 2016). Acetaldehyde is a highly volatile aldehyde formed from pyruvate or threonine catabolism (Ardö, 2006), which has been described as delivering a pungent, fruity (green apple) flavor with sweet notes (Mahattanatawee et al., 2005). High level of acetaldehyde found after fermentation of a malt-based beverage with L. plantarum NCIMB 8826 positively contributed to the acceptance of the beverage (Salmerón et al., 2015). Amino acids play a central role as flavor-forming substrate in LAB (Gänzle et al., 2007). Besides possessing taste properties of their own (e.g., sweet, bitter, sulfurous, and umami) (Solms, 1969), amino acids serve as substrate for Maillard reactions that can accumulate organoleptic-active carbonyl compounds, heterocycles as well as melanoidins (Pozo-Bayón et al., 2006). The by-products of amino acid catabolism in LAB have been repeatedly reported as important flavor-active compounds in liquid cereal-based fermentations (Coda et al., 2011; Mugula et al., 2003; Muyanja et al., 2012). Aldehydes and alcohols can be released from the catabolism of the amino acids, after conversion into α-ketoacids by means of aminotransferases, and subsequent decarboxylation into aldehydes (Ardö, 2006). The reduction of these compounds into alcohols, however, was often assigned to endogenous yeasts present in the raw cereals (Muyanja et al., 2012). Microbial fermentation can lead to the activation of the endogenous proteinases (pH optimum between 4 and 5 in wheat, rye, and barley) (Belitz et al., 2009), but LAB can also actively increase the fermentable nitrogen level providing proteases and peptidases (Coda et al., 2012; Thiele et al., 2002). Finally, free fatty acids such as oleic and linoleic acid can act as precursors for methylketones, alcohols, and lactones (Smit et al., 2005). However, because of the generally low-lipolytic activities of LAB, these volatiles are formed by other microorganisms associated with food production, e.g., molds in cheese. Because of the relatively low concentration of lipids in cereals, volatiles derived from lipolysis metabolism have not been studied in detail during liquid cereal fermentations (Peyer et al., 2016).

10.3 YOGURT-LIKE BEVERAGES MADE WITH OAT

Among the cereal grains, oat is known to provide a vast range of health benefits, including reduced symptoms of diabetes and obesity (Tapola et al., 2005; Zdunczyk et al., 2006). Oat is one of the major sources of β-glucan, a cell wall polysaccharide recognized as the main functional component of cereal
fiber and primarily responsible for these health benefits (Angelov et al., 2006). β-Glucan content in oat is about 2.3%–8.5% (Flander et al., 2007) and its structure consists of consecutively linked (1/4-β-d-glucosyl residues in oligomeric segments that are separated by single (1/3)-linkages along the polymer chain backbone (Lazaridou et al., 2004). According to the definition of the American Association of Cereal Chemists, oat β-glucan can be considered as dietary fiber and therefore contribute to “promote beneficial physiological effects including laxation and/or blood cholesterol attenuation and/or blood glucose attenuation” (AACC, 2001; Rasane et al., 2015). The health effects of oat β-glucan consumption were officially recognized first by the US Food and Drug Administration (US FDA, 1997) and more recently by the European Food Safety Authority (EFSA, 2010) according to which a daily consumption of 3 g of β-glucan leads to the reduction of blood plasma cholesterol concentration, which is a major risk factor for the development of coronary heart disease (Lazaridou et al., 2014). Moreover, oat β-glucan has potential anticarcinogenic property, as it reduces compounds that are causative agents of colon cancer (Butt et al., 2008), reduces blood cholesterol levels (Ripsin et al., 1992; Amundsen et al., 2003), and decreases blood pressure (Maki et al., 2007).

Due to all these benefits, oat has been widely exploited for making beverages in recent years, particularly, as a fermented beverage with functional properties (Marsh et al., 2014; Corbo et al., 2014). Moreover, with its high content of dietary fibers, including β-glucan, inulin, and resistant starch, oat is a good source of fermentable carbohydrates for colonic bacteria, which can act also as prebiotics in association with probiotic microorganisms (Gibson et al., 1995; Charalampopoulos et al., 2002b). As previously shown, probiotic and prebiotic effects might be additive or even synergistic. Their combination could benefit the host by improving survival and implantation of microorganisms in the gastrointestinal flora, by selectively stimulating the growth or activity of health-promoting bacteria in the intestinal tract, and by improving the gastrointestinal tract’s microbial balance (Roberfroid, 2000).

For all these reasons, probiotic fermented beverages are one of the most important categories of the functional food segments (Sirò et al., 2008). The attractiveness and high consumer acceptance of fermented functional beverages relies on the activity of specific living microorganisms, which is the main reason for their functionality (Gobbetti et al., 2010). Indeed, probiotic bacteria such as Bifidobacterium spp. and LAB have been used as starter cultures for the production of fermented oat beverages, delivering microorganisms to the gut and providing enhanced nutritional profile (O’Connor et al., 2005).

### 10.3.1 OAT-BASED PREBIOTIC, PROBIOTIC, AND SYMBIOTIC BEVERAGES

The nutritional properties of oat and the physiological characteristics of specific strains of LAB and bifidobacteria make oat probiotic yogurt-like beverages a suitable contributor to enhancing and maintaining health and improving the quality of life. Recently, several studies have shown the positive effect of oat fermentation by probiotic strains. For probiotic products, viability at a minimum of 6 log cfu/mL and activity of the bacteria are important parameters, as they must survive in the food during shelf life and during transit through the acidic conditions of the stomach in order to perform their beneficial effects (Tamime et al., 2005). Several probiotic species of different origin have been successfully grown in oat substrate, such as Lactobacillus rhamnosus are able to grow up to 7 log cfu/g and dominate in oat bran fermentation and L. plantarum in whole-grain fermentation, reaching viable cell counts of 10 log cfu/mL after 24 days of refrigerated storage (Loponen et al., 2007). In the latter case, the final result was a symbiotic product, combining the positive effects of probiotics with the oat prebiotic β-glucan, increasing the potential health benefits (Angelov et al., 2006). A symbiotic interaction
between prebiotics, such as β-glucan, and probiotics might result in increased health benefits for the host, by promoting the growth of the bacteria and releasing low-molecular fatty acids (butyrate, acetate, and propionate). The release of short-chain fatty acids can provide energy and acidify the bowel thereby producing a less advantageous environment for pathogens, and at the same time exert potential anticarcinogenic effect (Salminen et al., 1998; Gallaher, 2000). Therefore, the presence of both β-glucan and probiotic during gastrointestinal digestion is a strategy to further enhance the health attributes of oat beverages. The fermentation of oat-based substrates with Lactobacillus reuteri, L. acidophilus and Bifidobacterium bifidum delivered a yogurt-like product with acceptable appearance and taste and small repercussion on the β-glucan content after storage (Mårtensson et al., 2001). Similarly, a probiotic strain of L. plantarum was used to produce a functional oat drink having β-glucan content of 0.31%–0.36%, which remained unchanged throughout fermentation and storage (Angelov et al., 2005).

Symbiotics can have effect in the reduction of osteoporosis and improvement of mineral absorption (Scholz-Ahrens et al., 2007). Oat-based symbiotic yogurt-type beverage containing different LAB or bifidobacteria have been further developed and improved in the last years (Table 10.1). An oat yogurt containing L. plantarum, Lactobacillus paracasei ssp. casei, and L. acidophilus was developed at the University of Vermont, USA, and was shown to have some epithelial adhesion to Caco-2 cell lines indicating potential for intestinal colonization (Gokavi et al., 2005). In another case, an oat symbiotic yogurt-like beverage (Oagurt) was developed using probiotics (L. acidophilus, Lactobacillus casei, and Bifidobacterium), fortified with inulin to increase soluble fiber, minerals, and vitamins, and to which prepolymerized whey protein was added to improve the textural properties (Walsh et al., 2010). More recently, a yogurt-like beverage made with oat flakes and fermented by L. plantarum showed an increase in the polyphenols' availability and antioxidant activity as well as reduced hydrolysis index in vitro, highlighting the high-nutritional potential of these beverages (Nionelli et al., 2014).

An example of the market relevance of oat functional beverage is represented by Proviva (Skane Dairy, Sweden) and Yosa (Bioferme, Finland), deriving from wholegrain fermented oat, similar to flavored yogurt or porridge, containing L. acidophilus and B. bifidum. Yosa content of oat fiber and probiotic combines the effect of β-glucan on cholesterol reduction and the effect of LAB benefits to maintain and improve the environment in the intestinal balance of the consumer (Blandino et al., 2003; Salovaara, 1996). The potential beneficial effects of functional oat beverages on human health encourage further research for its complete exploitation in clinical usages.

10.4 YOGURT-LIKE BEVERAGES MADE WITH OTHER CEREALS: FROM TRADITIONAL TO INNOVATIVE PRODUCTS

On a worldwide basis, maize, rice, and wheat are the prevailing crops in terms of area reserved for cereal cultivation and total cereal production (Poutanen, 2012). However, ancient and/or minor cereals, such as kamut, spelt, einkorn, millet, and sorghum, and pseudocereals, such as quinoa, amaranth, and buckwheat, have generated great interest, particularly in Western countries, because of their higher content in beneficial minor components (dietary fiber, resistant starch, minerals, vitamins, phenolic compounds) (Coda et al., 2014) compared to staple grains (wheat, maize, rice), and the possibility to fulfill specific dietary needs, such as low gluten or gluten free (Zannini et al., 2012).

Cereals are considered a suitable substrate for the production of probiotic products (Kandylis et al., 2016). In addition, their consumption has been associated with the reduction of the risk of several
chronic diseases (Kandylis et al., 2016). For these reasons, besides several probiotic beverages that are being produced worldwide, nowadays the scientific community and the food industry try to produce innovative beverages based on single or multicereals (Kandylis et al., 2016) (Table 10.1). In many traditional cereal-based beverages (Table 10.1), popular in tropical regions of Asia and Africa, the grains are often heated, mashed, and sometimes filtered (Marsh et al., 2014). Not all the traditional cereal-based beverages are characterized by a yogurt-like texture. For the inoculation, the backslopping procedure is quite common, but the microbial populations responsible for the fermentation of these beverages are not well characterized (Marsh et al., 2014). Boza, consumed in Bulgaria and Turkey, is generated through the fermentation of a variety of cereals including barley, oat, rye, millet, maize, wheat, or rice, with the specific composition affecting the viscosity, fermentability, and content of the final beverage (Akpinar-Bayizit et al., 2010). The cereal is boiled and filtered, a carbohydrate source is added, and the mixture can be left to ferment spontaneously or with the use of backslop. *S. cerevisiae, Leuconostoc mesenteroides,* and *Lactobacillus confusus* were often isolated from Boza (Zorba et al., 2003). Togwa, a sweet and sour, nonalcoholic beverage, is one of the better studied African cereal beverages. This is produced from the flour of maize, sorghum, and finger millet and, sometimes, cassava root. The chosen substrates are boiled, cooled, and fermented for approximately 12 h to form a porridge, which is then diluted to drink (Kitabatake et al., 2003). Mahewu is similar in that maize or sorghum meal is fermented with millet or sorghum malt, and is available commercially (Mugochi et al., 2001). Bushera is generally prepared from germinated or nongerminated sorghum grains, and fermented for 1–6 days (Muyanja et al., 2003). These beverages are often used to wean children and also as a high-energy diet supplement. Koko sour water is the fermented liquid water created in the production of the fermented porridge koko. This contains a high LAB density and is used by locals to treat stomachaches and as a refreshing beverage (Lei and Jakobsen, 2004). Kvass is a fermented rye bread beverage common in Russia, which has seen much commercial success. The beverage can have a sparkling, sweet or sour, rye bread flavor (Jargin, 2009). Amazake is a sweet fermented rice beverage that is the nonalcoholic precursor to sake, produced in Japan. Steamed rice is mixed with rice-koji (*Aspergillus* mycelia and rice) and water, and is heated to 55–60°C for 15–18 h. Enzymes break down the rice and form glucose content of approximately 20%. Amazake is highly nutritious and is consumed for its purported health benefits (Yamamoto et al., 2011). Pozol, common in southeastern Mexico, has quite a peculiar method of production, in which maize grains are heat-treated in an acid solution, ground, and shaped into dough balls. These are then wrapped in banana leaves and fermented for 2–7 days, successively diluted with water and consumed as a beverage. Pozol hosts a variety of microorganisms including LAB, non-LAB, yeasts, and other fungi (Ben Omar and Ampe, 2000). Rice is a common substrate for the production of beverages especially in Asia and South America. Haria is a rice-based ethnic fermented beverage of East-Central India. It has low alcohol content of 2–3% (v/v) and titratable acidity of 1.42% (Ghosh et al., 2014). During fermentation yeast, mold, LAB, and *Bifidobacterium* spp. are present and through synergistic actions define the final characteristics of haria. They convert starchy materials of rice to malto-sugars and enrich the final product with antioxidants and bioactive substances (Ghosh et al., 2014). Similar characteristics were observed in another rice-based beverage of North Western Himalayas in India, the chhang (Thakur et al., 2015). A rice-based beverage with the name chicha was also produced in Brazil by Umutina Brazilian Amerindians (Puerari et al., 2015). LAB and *Bacillus* spp. were the dominant microorganisms in the beverage. The final product has no ethanol but glycerol and low acidity and is characterized as an acidic nonalcoholic beverage (Kandylis et al., 2016).
A yogurt-like beverage was produced using emmer (*Triticum dicoccum*) flour and a *L. plantarum* strain isolated from the same matrix and selected on the basis of different protechnological properties (Coda et al., 2011) (Fig. 10.1). The substrate, including 30% of emmer flour in water, was gelatinized prior to inoculation (Coda et al., 2011). The beverage texture was improved using an EPS-producer LAB strain, and it was found that the matrix allowed a proper survival of a probiotic *L. rhamnosus* during long-time storage at 4°C. Several nutritional properties, including a low-hydrolysis starch index, high-phytase activity, and high concentration of essential amino acids characterized the novel beverage (Coda et al., 2011). A beverage produced with malt and *L. acidophilus* showed high acceptance from the consumers, also due to high-acetaldehyde concentrations and mild pH values (Salmerón et al., 2014, 2015). Recently, cereal (rice, barley, emmer, and oat) and soy flour and grape must were used for making vegetable yogurt-like beverages (Coda et al., 2012). Two selected strains of *L. plantarum* were used for lactic acid fermentation, according to a process that included the flour gelatinization. All the

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**FIGURE 10.1**

Protocol for making a yogurt-like beverage using emmer (*Triticum dicoccum*) flour, as proposed by Coda et al. (2011). A pool of LAB strains, selected on the basis of protechnological, probiotic, and EPS-production properties, was used as starter for fermentation.
yogurt-like beverages had values of pH lower than 4.0 and both the starters remained viable at c. 8.4 log cfu/g throughout the storage. During fermentation, LAB utilized glucose, fructose, and malic acid, which were supplied through grape must (Coda et al., 2012). Compared to control beverages made without bacterial inoculum, an increase of total free amino acids was found during fermentation and storage. Also the concentration of polyphenolic compounds and ascorbic acid was higher when the LAB were used as starters, consequently leading to high antioxidant activity (Coda et al., 2012). Among the different cereal substrates, the beverages made with the mixture of rice and barley or emmer flours appear to possess the best combination of textural, nutritional, and sensory properties (Coda et al., 2012).

From the technological perspective, the influence of containers, substrates, metabolites, and fermentation kinetics, together with the peculiar microbiota of the traditional beverages, are the key factors to define the role of the different microorganisms in fermentation processes, and their contribution to the final features of the products (Marsh et al., 2014). At the same time, the characterization of the nutritional and functional properties is fundamental for commercialization and is required by the consumers. Nevertheless, the trend of the scientific community and the food industry is to use traditional beverages as a model for the development of new products, since the evaluation of the functional and sensory properties in controlled fermentations is easier (Marsh et al., 2014).

10.5 FUTURE TRENDS

Cereals are a very good alternative to dairy and can have multiple beneficial effects, which make them very suitable for functional beverages. Fermented cereal beverages have high-mineral content, and generally a lower-fat content than their dairy-based counterparts. They also naturally provide plant functional components, such as fiber, vitamins, minerals, flavonoids, and phenolic compounds, which can affect oxidative stress, inflammation, hyperglycemia, and carcinogenesis (Wang et al., 2014). The development of functional fermented-cereal products through application of defined LAB meets the current demand for healthier and diversified foods (Peyer et al., 2016) although the research in the area of nondairy probiotic beverages is currently in an early stage (Kandylis et al., 2016). The selection of specific microbial starters will be crucial to deliver specific healthy properties but also to reproduce the desirable characteristics of traditional health-promoting beverages in industrial scale (Marsh et al., 2014). The full prediction of the final sensory attributes, for example, is still a very complex task due to dynamic interactions between starter cultures, substrate, and fermentation conditions. The use of a broader array of cereal substrates inoculated with single or combined starters could be investigated under different conditions (Peyer et al., 2016), since the process conditions can be modulated to obtain a large range of specific effects on the sensory, technological, nutritional, and functional aspects of the final product.

Therefore, different aspects must be taken into account for the design of novel cereal-based yogurt-like beverages, at scientific and industrial levels, such as the characterization and the standardization of the bioactive compounds, the selection of starters able to produce bioactive compounds and to positively affect the organoleptic and texture features, the survival of probiotic organisms during storage, the application of natural biopreservatives to improve the natural image of the functional beverages, the bioavailability and metabolism of functional ingredients, the safety aspects related to the consumption, and the formulation of value-added products based on traditional fermented beverages (Corbo et al., 2014).
Nevertheless, the future of functional yogurt-like beverages, including their commercial success, mainly depends on the unequivocal demonstration of their efficacy in promoting health (Corbo et al., 2014). Thus, the cooperation between the food industry and the scientific community is strictly necessary to provide scientific evidence of the health claims, to improve the biotechnological tools for their large-scale production and distribution, and to enhance their attractiveness and sensory properties.

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CHAPTER 11

PREPARATION OF FUNCTIONAL YOGURT ENRICHED WITH OLIVE-DERIVED PRODUCTS

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11.1 INTRODUCTION

Healthiness is one of the most frequently mentioned reasons behind food choices in EU countries. In addition to traditional healthiness, food may contain specific components with a positive impact on health and because of this foods are considered as functional. Fermented foods constitute an important part of our food. Several potential claims are available on proposed beneficial health effects of fermented dairy foods including yogurt. Milk and yogurt constitute two of the most popular dairy products with high nutritional value, which are proven as successful matrices for the development of various health-promoting functional foods (Rowan et al., 2005). In recent years, a variety of dairy products supplemented with probiotic bacteria and/or bioactive components have been introduced to the market. Because of their health-promoting benefits, there has been an increasing interest in the development and consumption of new functional dairy products.

One category of bioactive components with officially recognized health-protecting properties is olive polyphenols (EFSA, 2011). This category of components appeared as an attractive candidate that could be used for the enrichment of dairy products and increase their health-protecting properties (Zoidou et al., 2014). Oleuropein (Fig. 11.1) comprises the major constituent of Olea europaea L. leaves and unprocessed olives (Bianco and Uccella, 2000; Soler-Rivas et al., 2000).

The olive leaf extract has been recognized by the European Medicines Agency (EMA) as an official herbal drug displaying a broad variety of health beneficial properties (EMA monograph). Many in vivo and in vitro studies have indicated that oleuropein exhibits a wide variety of biological activities, including antimicrobial, antioxidant, antiischemic, antihypertasic, anticoagulant, hypolipidemic, and antitumor properties (Hamdi and Castellon, 2005; Obied et al., 2005; Andreadou et al., 2006; Coni et al., 2000; Tassou and Nychas, 1995; Giamarellos-Bourboulis et al., 2006). Thus this natural product has attracted significant scientific and research interest, mainly connected with its potential protective role against infections and diseases, as well as the risk of developing breast, prostate and colon cancers, cardiovascular diseases, and diabetes. Most significantly, according to the scientific opinion of the European Food Safety Authority (EFSA, 2011), oleuropein (as hydroxytyrosol derivative) has been related to the protection of low-density lipoprotein from oxidation. The bioavailability of oleuropein has been studied (de Bock et al., 2013). It is absorbed after oral administration and is metabolized mainly to hydroxytyrosol, which is also a powerful antioxidant.

Although oleuropein is an abundant constituent of unprocessed olives, in most edible olives it is removed during the debittering process, resulting in a significant decrease in its nutritional intake. We
have identified a Greek edible olive variety (*Throuba thassos*), which is particularly rich in oleuropein (Zoidou et al., 2010), and based on this study we determined an acceptable and safe level for the daily nutritional uptake of oleuropein. In this frame, oleuropein appeared as a component that was worth studying for the enrichment of dairy products (Zoidou et al., 2014).

However, pure oleuropein is a relatively expensive ingredient and for this reason we also investigated, and present herein, the use of olive leaf extract as an alternative cheap source with a high concentration of oleuropein. Oleuropein is available as a nutraceutical in the world market mainly as a constituent of olive leaf extract, but until now there are no food preparations with this molecule. The olive leaf extract, besides oleuropein, contains several other bioactive compounds and exhibits antioxidant, antiinflammatory, antiviral, immuno-modulatory, and hypocholesterolemic effects (EMA monograph). Olive leaf has been in the focus of research interest because of its health beneficial effects especially in the treatment and prevention of several chronic diseases. In our study, we used the olive leaf extract in the form of an infusion. It should be noted that the recognized traditional use by the EMA concerns herbal tea in a decoction or infusion form.

Based on these data, we investigated the addition of oleuropein, either in pure form or in the form of dried olive leaf infusion, in milk and yogurt for the production of a new type of functional food.

One of the major challenges that had to be faced during this study was the impact of oleuropein on the taste and texture of yogurt. Since consumers demand products combining increased health benefits with good taste, the bitter taste of oleuropein constituted a serious drawback that had to be investigated. The questionable stability of oleuropein during processing comprised a second barrier that had to be studied. For this purpose, it was necessary to develop new analytical methods for the measurement of oleuropein in leaves, in the infusion, in milk, and in yogurt. Thus the development of functional dairy products based on oleuropein was an intriguing case.

### 11.2 MATERIALS AND METHODS

#### 11.2.1 CHEMICALS

All solvents and distilled water used throughout the experiments were obtained by Merck (Darmstadt, Germany) and were of high-performance liquid chromatography (HPLC) grade. All mobile phases were vacuum filtered through a 0.2 μm membrane filter (Scientific Resources, Eatontown, NJ, USA) and degassed in an ultrasonic bath prior to HPLC analysis. Pure oleuropein was purchased from Extrasynthese (Genay, France).
11.2.2 OLIVE LEAF SELECTION

The olive leaf selection was based on its oleuropein content. Olive leaves from 10 different varieties and geographic origins from Greece were selected and analyzed for their oleuropein content. The finally selected olive leaves originated from wild trees growing near Lake Volvi (northern Greece). More specifically, dry olive leaves in powder form (100 mg) were extracted with 20 mL of MeOH in a supersonic bath for 45 min. The supernatant was separated by centrifugation at 4000 rpm for 3 min. A part of the supernatant methanol extract (10.0 mL) was mixed with 0.5 mL of internal standard solution (0.5 mg syringaldehyde/mL in acetonitrile) and evaporated under reduced pressure at 40°C.

11.2.3 QUANTITATIVE NUCLEAR MAGNETIC RESONANCE ANALYSIS OF OLEUROPEIN CONTENT IN OLIVE LEAVES

The dry extract was dissolved in 600 μL of CD$_3$OD. $^1$H nuclear magnetic resonance (NMR) spectra were recorded at 400 MHz. Thirty-two scans were collected and the spectra were phased corrected and integrated automatically using TOPSPIN. The quantitation was based on the integration ratio between the aldehydic proton signal of syringaldehyde at 9.75 ppm and the proton of oleuropein appearing at 5.91 ppm. A calibration curve of oleuropein was prepared at seven different concentrations ranging between 70 and 4500 μg in a tube. The solutions for the construction of the calibration curve were prepared by mixing appropriate volumes of a stock solution of pure oleuropein (1 mg/mL in MeOH) with 0.5 mL of the internal standard solution (0.5 mg syringaldehyde/mL in acetonitrile) and evaporation under reduced pressure at 40°C. The equation used for the quantification of oleuropein in a tube was $y = 0.512x + 0.0904$, with $r^2 = 0.995$.

11.2.4 OLIVE LEAF EXTRACT PREPARATION

An aliquot of 24 g of dried olive leaves was added to hot water (450 mL) for 30 min, according to the EMA monograph. The obtained infusion was lyophilized to afford a dry extract (5 g). The oleuropein content of the olive leaf-lyophilized extract was measured by NMR using the previously described method. More specifically, 15 mg of the lyophilized extract were mixed with 0.5 mL of the internal standard solution (0.5 mg syringaldehyde/mL in acetonitrile) and evaporated under reduced pressure at 40°C. The mixture was dissolved in 600 μL of CD$_3$OD and the $^1$H NMR spectrum was recorded at 400 MHz.

11.2.5 SELECTION OF OLEUROPEIN DOSAGE

The determination of oleuropein dosage was based on its quantity that corresponded to the average consumption of 15 olive drupes per day (as measured for Throuba thassos olives; Zoidou et al., 2010). For this purpose, oleuropein was added to the milk to achieve three levels: 0.1, 0.2, and 0.4 mg/mL. The organoleptic properties of the novel products were examined by a sensory panel, which evaluated their taste, color, and texture using a 1–10 scale, in accordance with Tamine and Robinson (2000). The respective results suggested to proceed with oleuropein concentrations of 0.1 and 0.2 mg/mL. Based on the measured oleuropein content of the lyophilized olive leaf extract, a respective amount of extract (0.43 mg/mL) affording 0.1 mg/mL of oleuropein was found to be acceptable and was further used.
CHAPTER 11 PREPARATION OF FUNCTIONAL YOGURT ENRICHED

11.2.6 EXPERIMENTAL PROCEDURE FOR THE PRODUCTION OF MILK AND YOGURT PREPARATIONS

Full-fat raw cow’s milk was used for all the experiments. Milk was first spiked with oleuropein and in a next step the content and stability of the contained oleuropein in milk and the produced yogurt were determined during their treatment. A second set of experiments was performed using olive leaf extract in the place of pure oleuropein.

11.2.7 PREPARATION OF MILK ENRICHED WITH PURE OLEUROPEIN: STUDY OF HEAT RESISTANCE AND STORAGE

The resistance of oleuropein during the heating of milk was examined first. In particular, 40 mg of pure oleuropein was added to 200 mL of raw milk (fat content 3.5%) and mixed. The milk was heated at 90°C for 5 min in a water bath under continuous stirring, and then milk was cooled immediately with tap water and put into sterilized glass bottles. Next, the milk was stored at 4°C for 7 days. The oleuropein content was determined immediately after its addition in milk as well as after the heat treatment and every 2 days during storage. The experiment was duplicated.

11.2.8 MANUFACTURE AND STORAGE OF YOGURT ENRICHED WITH PURE OLEUROPEIN

Pure oleuropein was added to 500 mL of cow milk (fat content 3.5%), mixed well, heated at 90°C for 5 min in a water bath, cooled to 43°C, and divided into equal quantities (2 × 200 mL) in two sterilized glass cups. Then, 3% (v/v) of yogurt culture was aseptically inoculated and mixed well, and the milk was incubated at 42°C for 4.5 h until its pH reached 4.45. The prepared yogurts were cooled, stored at 4°C, and their oleuropein content was determined immediately after inoculation and the production of yogurt, as well as every 2 days during their storage. Control yogurt was also manufactured using a similar procedure without the oleuropein addition step. The process was repeated twice using the two dosages selected (0.1 and 0.2 mg/mL).

11.2.9 MANUFACTURE AND STORAGE OF YOGURT ENRICHED WITH OLIVE LEAF EXTRACT

The milk was homogenized and heated. The addition of olive leaf extract to milk was done at 35°C. The amount of added extract was 0.43 mg/mL corresponding to 0.1 mg/mL of oleuropein. Then, the milk was heated at 90°C for 5 min, cooled to 42°C, inoculated with traditional Greek yogurt at 1.5%, poured into 100 g sterilized cups, and incubated to the desired pH (4.6). The yogurts were refrigerated immediately at 4°C for 21 days and analyzed at 7, 14, and 21 days of storage.

11.2.10 PH MEASUREMENT

During the refrigerated storage, the quality of the produced milk and yogurt was monitored by measuring the pH and determining their sensory characteristics. In particular, pH was determined using a Hanna model HI 98240 pH meter (Hanna Instruments, Woonsocket, RI, USA) and titratable acidity was determined by titrating 10 mL of a sample with N/9 NaOH and expressed as Dornic degrees (°D).
11.2.11 SENSORY ANALYSES

Samples were assessed for sensory properties for the following attributes: external aspects (color and dry/humid), flavor, texture, basic tastes (acidic, salty, and bitter), and after-taste (intensity and persistence). The sensory acceptability of the new products was assessed by a panel of five persons of the Dairy Laboratory staff. The products were served at 7–10°C, milk in glasses and yogurt in plastic cups, immediately after their preparation and after 7 and 21 or 35 days of storage at 4°C, respectively. In this respect, their taste, color, and texture were graded using a sensory scale of 1 to 10 as follows: 1–2 bad, 3–4 not satisfying, 5–6 good, 7–8 very good, 9–10 excellent, according to Tamine and Robinson (2000). Their overall acceptability was also determined. Because of the small number of the members of the sensory panel the results should be considered as preliminary.

11.2.12 PHYSICOCHEMICAL ANALYSES

The composition was evaluated by means of Milkoscan (Milkoscan FTIR 120, Foss Electric, Denmark).

11.2.13 RHEOLOGICAL ANALYSES

11.2.13.1 Firmness

Firmness (g) was measured by a Brookfield texture analyzer LFRA 1000 using a 2 cm acrylic cylinder probe. The test speed was fixed at 2 mm/s and the penetration depth was 13 mm. Sample temperature was 20°C. Firmness was defined as the force necessary to reach the maximum depth.

11.2.13.2 Viscosity

Viscosity (cps × 1000) was measured using a Brookfield RV, DV-II viscometer (Brookfield Engineering Laboratories Inc., Stoughton, MA, USA) with a Helipath (Spindle, 94) rotated at 2 rpm for 1 min. Sample temperature was 20°C.

11.2.13.3 Syneresis

Syneresis was measured by syneresis index and water-holding capacity (WHC).

11.2.13.4 Water-Holding Capacity

WHC was determined by a procedure adapted from Akalin et al. (2012). A sample of 20 g yogurt (YO) was centrifuged for 10 min at 5000 g at 20°C. The WHC was expressed as percentage (w/w) of yogurt mass after removing the whey expelled (WE) and defined as WHC% = 100 × (YO − WE)/YO.

11.2.13.5 Syneresis Index

Syneresis index was measured according to the method described by Harwalkar and Kalab (1983) by emptying the yogurt from a cup (100 mL) into a filter, cutting crosswise into four pieces, draining for 24 h at 20°C, and collecting the amount of whey drained off in a graduated cylinder. The relative volumes (V%) of whey drained at 5 min and at 1 min increments thereafter for up to 3 h were plotted against time. The relative volumes at 3 h were estimated as the syneresis index.
11.2.14 QUANTITATION OF OLEUROPEIN IN MILK AND YOGURT

A method for the quantitation of oleuropein in milk and yogurt preparations was developed and validated. The proposed method included an extraction procedure of oleuropein and its quantitation through HPLC analysis.

The extraction procedure for milk was done with the addition of 2 mL of acetonitrile into 1 mL of milk under stirring for 2 min. Then, the mixture was centrifuged at 2000 rpm for 15 min and the supernatant was filtrated through a syringe filter pore 0.45 μm and injected for HPLC analysis.

The extraction procedure for yogurt was as follows: 1 mL of water was added to 1 g of yogurt and stirred for 2 min. Then, the mixture was centrifuged at 2000 rpm for 15 min and the supernatant was filtrated through a syringe filter of pore 0.45 μm. A portion of 20 μL was injected for HPLC analysis.

11.2.15 CHROMATOGRAPHIC INSTRUMENTATION AND METHODOLOGY

HPLC determination of oleuropein was carried out with a system consisting of a Finnigan Spectra system P4000 quaternary pump coupled to a Finnigan Spectra system UV6000LP diode array detector. For the chromatographic separation a Li Chromosorb C18 reversed-phase column (250 x 4.0 mm, ID 5 μm) equipped with a C18 Li Chromosorb precolumn was used.

The gradient elution program was implemented using a system of two solvents. Solvent A was a 2% w/w solution of acetic acid and solvent B was acetonitrile. The flow rate was constant at 1 mL/min and the total chromatographic analysis time was 40 min at ambient temperature. The following gradient program was applied: linear gradient 100%–95% of solvent A for 2 min, 95%–75% of solvent A from 2 to 10 min, 75%–60% of solvent A from 10 to 20 min, and 60%–50% of solvent A from 20 to 30 min. Then, 50% rate of solvent A from 30 to 34 min, which followed a linear gradient to 100% from 34 to 40 min. The injection volume was 20 μL. Oleuropein was determined at 254 nm at a retention time of 17 min. For the quantitation of oleuropein in the extracts, standard solutions of milk and yogurt spiked with oleuropein were prepared. Identification of the eluting peaks was performed by comparing their retention time values (tR) and the corresponding UV spectra (obtained from the diode array data) with those of the standards. The area of the peak was estimated by Qchromquest V.2.51 software. The oleuropein content of extracts was estimated from the calibration curve of the milk- and yogurt-spiked standards.

11.2.16 STANDARD SOLUTIONS

A stock solution of 1 mg/mL oleuropein in methanol was prepared and diluted in water to produce standard solutions of 0.02, 0.04, 0.1, 0.2, 0.4, and 0.6 mg/mL oleuropein concentrations. Milk and yogurt standards were prepared to provide 0.02, 0.04, 0.1, 0.2, 0.4, and 0.6 mg/mL concentrations of oleuropein by diluting appropriate volumes of the stock standard solutions in milk and yogurt. Stock solution was stored in a refrigerator and used for the preparation of standard solutions of milk and yogurt immediately prior to the analyses.

11.2.17 METHOD VALIDATION

The method was evaluated by measuring the linearity, precision, relative standard deviation percent (RSD%), accuracy, relative percentage error percent (Er%), and determining the limits of detection and
quantitation (LOD and LOQ). Moreover, a reproducibility study (system precision) was also performed. For the linearity study, six milk and yogurt standards containing oleuropein 0.02, 0.04, 0.1, 0.2, 0.4, and 0.6 mg/mL were analyzed four times and the data linearity was verified through linear least-squares regression analysis. In addition, the intraday and interday (for 5 different days) precisions were also determined along with the RSD%. The accuracy was estimated by analyzing three milk and yogurt standards at four concentration levels of 0.02, 0.10, 0.20, and 0.4 mg/mL or g. The results were expressed as Er%, defined as \([\text{assayed concentration} - \text{true concentration}] / [\text{true concentration}] \times 100\). For the recovery calculation, milk and yogurt standards and standard solutions containing 0.02, 0.1, 0.2, 0.4, and 0.6 mg/mL or mg/g of oleuropein were analyzed in accordance with the proposed extraction procedure. The recovery was determined in respect to the experimental response values as the ratio of the peak area for oleuropein in the milk and yogurt standards against that of the standards. The LOD and LOQ were determined by measuring the background response, and running six blank samples of milk and yogurt at maximum sensitivity. The signal-to-noise ratio of 3:1 (peak area ratio of the oleuropein vs. baseline noise) and 10:1 were used for the calculation of the LOD and LOQ, respectively. The reproducibility study was performed by injecting a standard of 0.5 mg/mL in five replicates (n=5).

11.2.18 LIQUID CHROMATOGRAPHY–MASS SPECTROMETRY QUALITATIVE ANALYSIS

Ultrahigh-pressure liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) monitoring of oleuropein was performed on an Agilent 1290 Infinity UHPLC interfaced to a 6460 triple-quadrupole mass spectrometer with electrospray ionization (ESI) via Jet Stream Technology (Agilent Technologies, Santa Clara, CA, USA). The UHPLC was equipped with a binary pump with an integrated vacuum degasser (G4220A), an autosampler (G4226A) with thermostat (G1330B), and a thermostatted column compartment (G1316C). The samples were analyzed using a Poroshell 120 EC-C18 column (2.1 × 150 mm 2.7 μm, Agilent Technologies). The mobile phase consisted of 0.1% formic acid in water (A) and acetonitrile (B) with the following gradient program: 0–2.5 min with 10% (B), 3–6 min 25% (B), 7.5 min 40% (B), and 8.5–9.5 min 95% (B). The flow rate was 0.4 mL/min, and the injection volume was 1 μL.

Negative ESI mode was used. The drying gas temperatures and flow rate were 250°C and 8 L/min, respectively. The sheath gas temperature and flow rate were 350°C and 11 L/min, respectively. The nebulizer gas pressure, capillary voltage, and dwell time were 45 psi, 3.5 kV, and 200 ms, respectively.

Total ion as well as multiple reaction monitoring mode was utilized to confirm the identity of oleuropein and the peak purity. Precursor and product ions were identified and optimized using a MassHunter Optimizer (Agilent Technologies).

Oleuropein was monitored through the fragmentation of the precursor ion 539.2 to the product ion 275.1 using a fragmentor voltage of 165, collision energy of 20, and retention time of 6.3 min.

11.2.19 MICROBIOLOGICAL ANALYSIS

The total viable microflora in milk and yogurt was enumerated by the pour-plate method using plate count agar (Merck, Darmstadt, Germany). The plates were incubated at 30°C for 72h (IDF 100B:1991) and microbiological count data were expressed as log_{10} of colony forming units (cfu) per mL or gram. Yogurt lactic acid bacteria (\textit{Streptococcus thermophilus} and \textit{Lactobacillus delbrueckii} ssp. \textit{bulgaricus})
counts were estimated according to the IDF standard 117, 2003 and yeasts and molds were counted according to IDF standard 94, 2004. The enumeration was performed on milk or yogurt just after the inoculation with oleuropein or olive leaf extract, and then after 4 and 7 days for milk and after 14 and 21 or 35 days for yogurt at 4°C. All determinations were made in duplicate.

11.2.20 STATISTICAL ANALYSIS
Data were subjected to analysis of variance using Software Statgraphics Plus for Windows v.5.2 (Manugistics, Inc., Rockville, MO, USA) to test the effect of the concentration of oleuropein and storage time on yogurt characteristics.

11.3 RESULTS AND DISCUSSION
11.3.1 HEALTH-PROTECTING PROPERTIES OF OLIVE LEAF EXTRACT AND OLEUROPEIN
According to the EMA monograph, olive leaf herbal tea is a safe traditional herbal medicinal product used to promote the renal elimination of water, in mild cases of water retention after serious conditions have been excluded by a medical doctor. According to the same monograph, herbal tea can be prepared either in the form of decoction or as an infusion.

Besides the officially recognized therapeutic indication, olive leaf extract has been found to possess a significant number of other health-protecting properties based on in vitro, in vivo, and clinical studies. More specifically, the EMA monograph reports several studies supporting that olive leaf can be used to enhance the immune system as an antimicrobial, antiviral, antioxidant, hypoglycemic agent, and also to protect from cardiovascular problems (antihypertensive, antiplatelet, hypolipidemic activity). Although these properties are not supported by sufficient clinical data the beneficial role of olive leaf on human health is well accepted.

Many of these properties have been related to oleuropein. Pure oleuropein has been found to have antimicrobial, antioxidant, antiischemic, antihypertasic, anticoagulant, hypolipidemic, and antitumor properties (Hamdi and Castellon, 2005; Obied et al., 2005; Andreadou et al., 2006; Coni et al., 2000; Tassou and Nychas, 1995; Giamarellos-Bourboulis et al., 2006).

Concerning the available toxicological data, it is known that the LD50 in mouse after oral administration is >3000 mg/kg and for oleuropein after intraperitoneal administration it is >1000 mg/kg. Although there are no available data for chronic oral toxicity, the safety profile of olive leaf extracts can be judged as good from the existing clinical data and from their long-term use (more than 30 years) in the European market.

For all these reasons, it was a very attractive proposition to incorporate oleuropein or olive leaf extract in dairy products to create a new type of functional food.

11.3.2 DOSE SELECTION
The selection of the appropriate dose for oleuropein incorporation in novel products was a critical point for this endeavor. Based on the quantity of oleuropein measured for the Throuba thassos variety (average 1.2 mg/drupe) (Zoidou et al., 2010) and considering that an intake of 15 olive
drupes per day is quite usual for the Greek population, total oleuropein intake could be estimated as approximately 20 mg per day. In addition, since the average consumption of dairy products corresponds to 200 mL milk or 200 g yogurt per day, we selected three different levels of oleuropein (0.1, 0.2, and 0.4 mg/mL) to incorporate into milk or yogurt (mg/g). These novel products were tested by the sensory panel immediately after their preparation, which found that the first two preparations exhibited acceptable sensory characteristics and were further investigated for their behavior during storage. On the contrary, milk and yogurt containing 0.4 mg/mL or mg/g of oleuropein produced a taste reminiscent of rancid oil and were only marginally acceptable, hence, they were not further studied.

Considering the high cost of pure oleuropein, we also investigated the use of olive leaf extract with standardized oleuropein content as an alternative cheap source of oleuropein. For this purpose it was first necessary to develop a rapid method for measuring oleuropein levels in leaves by quantitative NMR (qNMR). Then, after screening of several different olive varieties, we recognized a source of olive leaves with very high oleuropein content. Olive leaves of wild trees growing near Lake Volvi (northern Greece) were found to contain the highest oleuropein in the lyophilized infusion. Those trees constitute the largest wild olive orchard in Greece with a population of 100,000 trees. The leaves of those trees were used for the preparation of olive leaf infusion according to the EMA monograph. The infusion was lyophilized and the dried extract was analyzed for its oleuropein content by qNMR (Fig. 11.2).

![FIGURE 11.2](image)

\[^1\text{H} \] nuclear magnetic resonance (NMR) profile of the olive leaf extract showing the peak of oleuropein used for quantitation. I.S, internal standard.
The extract was found to contain 230 mg of oleuropein per gram (23% w/w). Based on this measurement we used a dose of 0.43 mg of extract per mL of milk, corresponding to 0.1 mg of oleuropein. The enriched milk was used next for the preparation of the enriched yogurt. Olive leaf extract standardized in oleuropein content can be easily prepared at a very low cost, much cheaper than pure oleuropein. The concentration used was found to be organoleptically acceptable and the enriched yogurt was further investigated for its physicochemical, rheological, and microbiological behavior during storage.

11.4 MILK ENRICHED WITH PURE OLEUROPEIN

11.4.1 QUANTITATION METHOD VALIDATION

The method was evaluated through the determination of oleuropein content in milk revealing good linearity, precision, accuracy, and reproducibility. All correlation coefficients calculated were higher than 0.990. In addition, the intraday precision, expressed as RSD%, ranged from 3.7% to 7.9% for the three concentration levels and was lower as compared to those of interday precision. The LOD and LOQ were 0.025 mg/mL and 0.074 mg/mL, while the recovery in the milk samples was 73%–93% (average 85%), indicative of a nearly quantitative recovery. The estimated accuracy values were within acceptable levels for oleuropein, while the system reproducibility was low (2.6%). All milk control extracts used in the experiments were oleuropein free, as determined by HPLC analysis.

11.4.2 MILK PROCESSING, HEAT RESISTANCE, AND STABILITY IN COLD STORAGE

According to quantitative HPLC-UV and qualitative LC-MS analysis, oleuropein content in milk was not affected by heat processing, indicating the industrial application potential of this molecule in yogurt manufacture. Oleuropein stability was also tested during the extended storage of milk at 4°C, proving that the molecule remained intact. Furthermore, no metabolites were detected in any screened UV wavelength, indicating that no decomposition of this compound occurred. It must be noted that according to previous reports and patents, the addition of some phenolics to milk prior to heat treatment enhanced its storage stability (Morgan et al., 1971). In addition, according to O’Connell and Fox (2001) the ability of various phenols to improve milk processability is attributed to their interaction with milk proteins. In particular, Sarker et al. (1995) reported that catechin, a green tea polyphenol, increases the volume and improves the foaming properties of β-lactoglobulin, while phenol-rich extracts or purified phenols such as caffeic acid markedly increase the heat stability of milk at 140°C (O’Connell and Fox, 1999).

11.5 YOGURT ENRICHED WITH PURE OLEUROPEIN

11.5.1 QUANTITATION METHOD VALIDATION

The HPLC-UV method was evaluated through the determination of oleuropein in novel yogurt preparation, displaying a linear relationship between oleuropein response (measured as peak area) and the corresponding concentration. The correlation coefficients were higher than 0.990 ranging from 0.997 to 0.9990. The data revealed good precision and accuracy with the lowest values corresponding to the lower quantities of oleuropein (0.02 mg/g). LOD and LOQ were 0.003 and 0.009 mg/g, respectively,
while recovery in the yogurt samples was close to 75%, the lower value obtained at the lower concentration.

All yogurt control extracts used in the experiments were oleuropein free, as determined by HPLC and LC-MS analysis.

11.5.2 YOGURT MANUFACTURE AND STORAGE

The fermentation period of the milk enriched with oleuropein was found to be normal (2.45 h), a value comparable to the control milk. Though oleuropein is considered as an antimicrobial agent, in this study it did not inhibit the growth of lactic acid bacteria when they were incubated together. In previous studies, it has been reported that oleuropein has an antimicrobial activity against brine lactic acid bacteria \textit{Lactobacillus plantarum}, \textit{Lactobacillus brevis}, \textit{Leuconostoc mesenteroides}, and \textit{Pediococcus cerevisiae} (Fleming et al., 1973). However, no data exist regarding the ability of oleuropein to inhibit the growth of yogurt bacteria.

According to HPLC results, the concentration of oleuropein determined by HPLC after 4.5 h incorporation in yogurt was similar to the processed milk. Fermentation for several hours did not cause any degradation to oleuropein, suggesting that this molecule was unaffected by starter bacteria. It must be noted that Giafardini et al. (1994) and Marsilio and Lanza (1998) studied the ability of an oleuropein degrading strain of \textit{L. plantarum} to grow in the presence of oleuropein. They found that in the absence of glucose, oleuropein incorporation in the cultivation medium caused complete degradation of derivative products, whereas in the presence of glucose oleuropein remained almost intact in the cultivation medium. This observation indicates that oleuropein is degraded unless there is another carbon and energy source such as glucose, which may be utilized more readily. McCue and Shetty (2005) also demonstrated that other phenolics were also not affected by yogurt microflora in the presence of kefir culture during yogurt production from soymilk.

Moreover, the acids produced during lactic acid fermentation did not affect oleuropein. After 4.5 h of storage the pH of yogurt containing oleuropein was 4.45, a value comparable to control, indicating that oleuropein neither supported nor impeded lactic acid production.

The stability of oleuropein was also tested during extended storage of yogurt at 4°C. Oleuropein was found to be chemically stable in acidic conditions up to 35 days of storage (a slight reduction after the 27th day was not considered as statistically important). Chemical stability was also confirmed using qualitative LC-MS analysis.

Concerning the bacterial population of the manufactured milk and yogurt during storage, the following data were collected. The average total viable microflora in raw milk was 4.78 log cfu/mL. After heat treatment, it was 2.20 log cfu/mL and after 4 and 7 days storage at 4°C it was 3.45 and 4.64 log cfu/mL, respectively. Viable microflora in the 0-day yogurts was 8.71 log cfu/g. After 14 days, the average counts dropped to 8.33 and at the end of the refrigerated storage they dropped to 7.52 log cfu/g. All yogurt samples, however, contained $10^7$–$10^8$ cfu/g for the entire period of 35 days. In general, there were no significant differences for total microflora among milk or yogurt samples with or without oleuropein.

11.5.3 PH MEASUREMENTS AND SENSORY CHARACTERISTICS

After manufacture and storage, the quality of the two products was evaluated by determining their pH values and sensory characteristics. Concerning the pH, a similar pattern was observed between the test and the control products, since the pH value was 6.45 in milk and was not markedly changed after
storage for 7 days at 4°C. In the case of fresh yogurts, the pH was 4.45 and diminished to 4.29 and 4.24 after 15 and 35 days of storage, respectively.

The sensory acceptability of the novel milk and yogurt was also tested immediately after their preparation and after 7 and 35 days of storage. The two selected concentration levels of oleuropein (0.1 and 0.2 mg/mL or g) gave preparations equally acceptable with the control ones ($P > .05$), since changes in yogurt taste were not observed (Table 11.1).

Many researchers have reported that the addition of phenolic compounds into dairy products alters their organoleptic properties. In some cases, phenols were also responsible for distinct off-flavors caused by protein interaction through Maillard reactions (Parks and Allen, 1973; Walker and Manning, 1976; Luck et al., 1994) or oxidation after the heat treatment of milk (Dumont et al., 1974), or even when they were added as flavoring agents (Maga, 1988). Thus the effect of phenols as functional ingredients on the quality of dairy products has been advocated and attributed to the protein–polysaccharide–phenols interaction. The extent of this interaction depends on the pH, the molecular properties of phenols, or the presence of specific polysaccharides. The enzymatic oxidation to quinones may also play an important role as well (O’Connell and Fox, 2001). The sensory scores of milk and yogurt prepared for the experiment, after 7 and 35 days of storage, respectively, were also determined. It should be noted that the mean scores for taste and color gradually decreased, while the mean scores for texture increased in yogurts as the storage progressed. Generally, the oleuropein-based yogurts were firmer than control yogurts. These changes in sensory characteristics were similar with the control samples. Nevertheless, all the products were acceptable to the sensory panel, characterized as “very good,” and none of them had any off-flavor.

### Table 11.1 Sensory Analysis of Yogurts Incorporated With Oleuropein During Storage at 4°C (Zoidou et al., 2014)

<table>
<thead>
<tr>
<th>Criteria</th>
<th>Storage Period (days)</th>
<th>Yogurt Code</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>Texture</td>
<td>1</td>
<td>8.01&lt;sup&gt;a&lt;/sup&gt;&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>8.33&lt;sup&gt;a&lt;/sup&gt;&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>35</td>
<td>8.70&lt;sup&gt;a&lt;/sup&gt;&lt;sup&gt;g&lt;/sup&gt;</td>
</tr>
<tr>
<td>Taste</td>
<td>1</td>
<td>7.11&lt;sup&gt;a&lt;/sup&gt;&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>7.07&lt;sup&gt;a&lt;/sup&gt;&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>35</td>
<td>6.18&lt;sup&gt;a&lt;/sup&gt;&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Color</td>
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<td>8.52&lt;sup&gt;a&lt;/sup&gt;&lt;sup&gt;f&lt;/sup&gt;</td>
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<tr>
<td></td>
<td>7</td>
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</tr>
<tr>
<td></td>
<td>35</td>
<td>8.04&lt;sup&gt;a&lt;/sup&gt;&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Total</td>
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<td>23.64&lt;sup&gt;a&lt;/sup&gt;&lt;sup&gt;f&lt;/sup&gt;</td>
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<td>23.69&lt;sup&gt;a&lt;/sup&gt;&lt;sup&gt;f&lt;/sup&gt;</td>
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<tr>
<td></td>
<td>35</td>
<td>22.92&lt;sup&gt;a&lt;/sup&gt;&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

A, B: yogurt with 0.1 and 0.2 mg/g oleuropein respectively; C: yogurt without oleuropein.

“a” means in row at the same storage time with a common superscript do not differ significantly ($P > .05$, LSD test).

“e, f, g” means in column at different storage time with a common superscript do not differ significantly ($P > .05$, LSD test).
11.6 YOGURT ENRICHED WITH OLIVE LEAF EXTRACT

Having the positive results from the study of yogurt enriched with pure oleuropein we proceeded to the next step where the yogurt was enriched with olive leaf extract. Following a similar method of manufacture and storage as in the first set of experiments we studied the changes in composition, pH, acidity, rheological, and microbiological and sensorial parameters during manufacture and storage. It should be noted that in the case of yogurt enriched with olive leaf extract we investigated more in depth the impact on rheological characteristics. Besides the health benefits related to the nutritional profile and the presence of live microorganisms, rheological characteristics play a very important role in sensory evaluation and in consumer acceptability. The most typical defects of yogurts are low viscosity and reduced firmness or syneresis and liquid consistency (Domagala et al., 2013), and for this reason the impact of the added extract on those specific factors was thoroughly studied.

11.6.1 YOGURT COMPOSITION, PH, AND ACIDITY

The average composition of 0-day yogurts (Table 11.2) revealed that the enrichment with olive leaf extract did not cause any difference in fat. However, total solids and protein content were higher in enriched yogurt compared to the control because of the addition of the extract.

The enriched yogurt also presented higher pH and titratable acidity values (Table 11.3) resulting in a buffering capacity effect. The titratable acidity and pH values had a normal variation during storage and after 21 days the pH was similar and within the acceptance range of 4.30–4.40 in both yogurts.

| Table 11.2 Composition of Control Yogurt and Yogurt Enriched With Olive Leaf Extract |
|-----------------|-----------------|-----------------|-----------------|
| **Product**     | **Fat (%)**     | **Protein (%)** | **Total Solids (%)** |
| Enriched yogurt | 3.70            | 3.30            | 12.50           |
| Control         | 3.60            | 3.05            | 11.65           |

| Table 11.3 pH, Acidity, and Rheological Parameters of Control Yogurt and Yogurt Enriched With Olive Leaf Extract During Storage at 4°C |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| **Product**     | **Days of storage** | **Control** | **Enriched Yogurt** | **Control** | **Enriched Yogurt** | **Control** | **Enriched Yogurt** | **Control** | **Enriched Yogurt** | **Control** | **Enriched Yogurt** | **Control** | **Enriched Yogurt** | **Control** | **Enriched Yogurt** |
| pH              | 1               | 7              | 14              | 21             | 1               | 7              | 14              | 21             | 1               | 7              | 14              | 21             | 1               | 7              | 14              |
| Acidity (°D)    | 4.38            | 4.31           | –               | 4.30           | 4.46            | 4.41           | 4.38           | 4.40           | 98              | 78              | 82              | 85              | 98              | 78              | 82              |
| Firmness (g)    | 113             | –              | –               | 138            | 128             | 151            | 159            | 169            | 128             | 151            | 159            | 169            | 128             | 151            | 159            |
| Viscosity (cps x 1000) | 360            | 420            | 380            | 440            | 460             | 460            | 460            | 460            | 460             | 460            | 460            | 460            | 460             | 460            | 460            |
| Water-holding capacity (%) | 49              | 41.88           | 44.01           | 49.6            | 42.83            | 45.24           |

Domagala et al., 2013
11.6.2 RHEOLOGICAL PARAMETERS
Texture is a very important characteristic of yogurts. It is closely linked with the yogurt inner structure, which finally determines the overall quality of the yogurt. The results of the rheological analysis of the yogurts are presented in Table 11.3. The effect of the extract used for the enrichment was significant for all the rheological parameters of yogurt evaluated.

11.6.3 SYNERESIS
Syneresis is the shrinkage of the gel, which then leads to whey separation (Lucey, 2004). Syneresis occurs because of the loss of the ability of the yogurt gel to retain all of the serum phase because of weakening of the gel network.

Syneresis index was estimated by the ability of fresh yogurts to retain the serum. Increased whey expulsion from both yogurts was observed with time (Fig. 11.3).

The separation proceeded in two stages. A rapid rate of whey loss within the first 5–10 min was followed by a slower rate for up to 30 min. During this period the rate of whey drainage was higher for the enriched yogurt. Next, the separated whey increased slowly and after 1 h the relative volume was 44% and 41% for the enriched and the control yogurts, respectively. Approximately 87% and 80% of the whey drained over the 24-h period was drained within 1 h. The syneresis was leveled off at 3 h and was the same (~50%) for both yogurts. The whey drained from the enriched yogurt was very clear while the other was cloudy.

Syneresis was also estimated by the WHC during all storage time. On the 0-day yogurts the WHC was 49%–49.5%. The WHC values decreased during storage. However, the enriched yogurt presented higher WHC than the control yogurt, 42.83% compared to 41.88% after 14 days and 45.24% compared to 44.01% after 21 days.

Casein micelles aggregate through isoelectric precipitation by the action of lactic acid bacteria. During storage the casein strands can be broken and syneresis occurs. Manufacturers attempt to prevent these defects by increasing the total solids content of milk by adding milk ingredients and stabilizers (Sodini et al., 2005; Domagala et al., 2013; Roumanas et al., 2016; Sakkas et al., 2016). Polyphenols have a significant affinity for proteins that lead to the formation of soluble complexes, which can grow in size and even form sediments. Most models suggest that protein–polyphenol complexes are formed.

![Figure 11.3](image-url)

**Figure 11.3**
Separation of whey by draining yogurt: (■) enriched with olive leaf extract and (♦) control.
by multiple weak interactions (mainly hydrophobic) between the amino acid side chains and the poly-
phenol aromatic rings, indicating that the association of polyphenols with proteins is mainly a surface
phenomenon. However, sometimes these interactions could be complemented by hydrogen bonding,
which reinforces and stabilizes the complexes (Charlton et al., 2002; Oliveira et al., 2001).

Sastry and Rao (1990) reported that the bonds between polyphenols and proteins are more effective,
promoting the formation of a protein network with smaller pores and greater ability to increase water-binding
capacities, and at low pH the dissociation of proteins have more binding sites. These polyphenol–casein
stable complexes, because of the interaction of protein and polyphenol, may be the reason for the improved
syneresis in enriched yogurts with polyphenols, as was estimated by WHC.

11.6.4 FIRMNESS AND VISCOSITY

Firmness and viscosity values increased for both yogurts during cold storage. Yogurts enriched with
olive leaf extract developed higher firmness and viscosity throughout the storage period. This positive
effect is highly dependent on the higher content of total solids, proteins, and on the type of proteins
(Oliveira et al., 2001). A higher protein rearrangement occurred in the enriched yogurt because of the
protein–phenolic compound interactions. The effect of these interactions on structural properties has
been illustrated by several studies (Ozdal et al., 2013). Sometimes these interactions create stable complexes
with stronger internal bonds maximal at the isoelectric point of the proteins (O’Connell and Fox,
2001) and increase also the molecular weight of the proteins (Rawel et al., 2005).

11.6.5 MICROBIOLOGICAL CHARACTERISTICS

Olive leaf extract is a known antimicrobial agent, so it was important to check the survival of yogurt
microorganisms in enriched yogurts (Chouchouli et al., 2013; O’Connell and Fox, 2001). It was found
that the enrichment of yogurt with olive leaf extract did not cause change in the populations of lactic
acid bacteria compared to the control, as also happened in the case of pure oleuropein. The counts of
yogurt bacteria increased in the first 7 days, then decreased during cold storage in a similar manner in
control and enriched yogurt indicating that the enrichment did not affect their viability. Both yogurts
presented a high number of lactic acid bacteria throughout storage above the recommended level of
10^7 cfu/g (Table 11.4) (Codex Alimentarius, 2011). Yeasts and molds were not found.

<table>
<thead>
<tr>
<th>Table 11.4 Bacteria During Storage of Control Yogurt and Yogurt Enriched With Olive Leaf Extract</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Product</strong></td>
</tr>
<tr>
<td>Days of storage</td>
</tr>
<tr>
<td>Thermophilic cocci</td>
</tr>
<tr>
<td>Thermophilic bacilli</td>
</tr>
<tr>
<td>Yeasts–molds</td>
</tr>
</tbody>
</table>

^a Streptococcus thermophilus.
^b Lactobacillus delbrueckii ssp. bulgaricus.
11.6.6 **ORGANOLEPTIC PROPERTIES**

Regarding the organoleptic properties, both yogurts showed no differences in texture. The addition of the olive leaf extract reflected a bitter taste and a slight sharp after-taste of olive leaf, which, however, did not change the natural yogurt flavor. It should be noted that a light green color was observed in the enriched yogurt.

11.7 **CONCLUSION**

As presented herein and as shown elsewhere (Zoidou et al., 2014), the addition of oleuropein in milk and yogurt can lead to novel foodstuff preparations. For the first time we demonstrate herein the possibility of using olive leaf extract as an alternative source of oleuropein leading to products with enhanced health-protecting properties. For this purpose it was first necessary to develop and validate two efficient methods for the reliable determination of oleuropein in milk and yogurt preparations. The results indicated that oleuropein is resistant during the heating of milk. During the coagulation of milk, oleuropein was not hydrolyzed by the produced acids or metabolized by lactic acid bacteria, nor was their growth inhibited. Oleuropein was completely soluble in the selected concentrations (0.1 or 0.2 mg/mL) without adding any peculiar taste or flavor, while its stability during milk and yogurt storage at 4°C was unequivocally proven. In the case of yogurt enriched with olive leaf extract, the growth of lactic acid bacteria and pH were similar to the control during cold storage. The enriched yogurt contained more total solids and proteins. The utilization of olive leaf extract in yogurt improved its rheological properties (higher firmness and viscosity and less syneresis). Since from a technological point of view the presence of oleuropein in milk does not have any negative effect in the yogurt manufacturing process and considering the significant biological value of oleuropein was proved repetitively by numerous research reports, it is concluded that this molecule can be added as an active ingredient in milk and yogurt preparations for the production of novel functional foods with significant health benefits. Finally, the olive leaf extract may become a new convenient yogurt ingredient because it appears to improve not only its health-protecting properties but also its rheological properties.

**ACKNOWLEDGMENT**

We would like to thank the company Volvi estate for providing the olive leaves.

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12.1 GENERAL INTRODUCTION TO YOGURT

12.1.1 YOGURT

Yogurt is a fermented product resulting from the growth of lactic acid bacteria (LAB), *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus*, in milk (Adams and Moss, 2000). There are different types of commercially available starter cultures, available as freeze-dried powders in moisture-proof packs. These packs, if frozen, can stay fresh for more than 2 years, while packs that are left in the refrigerator can be kept for up to 1 year.

Cow’s milk is the most commonly used milk for the preparation of yogurt, but the milk from water buffaloes, goats, horses, camels, and yaks can also be used where available locally (Donovan and Shamir, 2014). The aroma, taste, and texture of the yogurts may vary with the type of milk and milk-processing method used (Routray and Mishra, 2011; Sodini et al., 2004). Yogurt prepared at home can be made from either homogenized milk or nonhomogenized milk. However, in commercial practice, homogenized types of milk (skim, partly skimmed, whole) are most frequently used.

Whole milk contains the most solids and produces a thick, rich, and creamy yogurt. According to the United States Code of Federal Regulations (2006), yogurt should contain more than 8.25% milk solids-not-fat (SNF). When 2% skimmed milk is used for the preparation of yogurt, milk powder and unflavored gelatin need to be added to replace the solids that have been removed. Typically, milk powder is added to 2% skimmed milk prior to fermentation, at a rate of 62.5 mL per liter of milk.

Yogurt fermentation requires precise temperature control. Milk is first heated to 85°C for 30 min to denature the milk proteins, particularly the whey proteins. After heating, the milk is allowed to cool to about 45°C and a starter culture consisting of *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus* is added. The temperature is maintained between 40 and 43°C for 4–6 h until the fermentation is complete (Adams and Moss, 2000).

Lactose-intolerant individuals may choose yogurt rather than other dairy products, since some lactose has been converted to lactic acid by the starter bacteria present in the yogurt (Shiby and Mishra, 2013). Lactic acid, a weak organic acid, gives yogurt its sourness and acts on the milk proteins to produce its unique textural characteristics. The natural sourness of yogurt is sometimes offset by adding sugar, flavor, or fruit, or by placing fruit or fruit jam at the bottom of the container.
12.1.2 LACTIC ACID BACTERIA IN YOGURT

Yogurt cultures of *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus* are most commonly used. Other *Lactobacillus* spp. such as *Lactobacillus acidophilus* and *Lactobacillus casei*, and *Bifidobacterium* spp. such as *Bifidobacterium longum* and *Bifidobacterium bifidum*, are also sometimes added during or after culturing yogurt to maintain a higher population of live probiotic organisms in yogurts. *L. delbrueckii* ssp. *bulgaricus*, a subspecies of *L. delbrueckii*, belongs to the genus of *Lactobacillus* and family of Lactobacillaceae. It is a Gram-positive rod that may appear long and filamentous. It is neither motile nor spore forming. This organism is regarded as aciduric or acidophilic, since it requires a low pH (∼5.4–4.6) to grow effectively. *L. delbrueckii* ssp. *bulgaricus* has complex nutritional requirements, growing on a limited number of carbohydrates and requiring pantothenic acid and niacin (Kandler and Weiss, 1986; Hammes and Vogel, 1995).

*S. thermophilus* is also classified as a LAB; it belongs to the genus *Streptococcus*, family of Streptococcaceae. This organism is a homofermentative facultative anaerobe and a thermophilic Gram-positive bacterium with an optimal growth temperature at 45°C. It tests negative for cytochrome, oxidase, and catalase activities, and positive for alpha-hemolytic activity. It is neither motile nor capable of forming spores (Kandler and Weiss, 1986). *S. thermophilus* is capable of generating energy in the form of ATP by aerobic respiration with the presence of oxygen; however, without the presence of oxygen, it can still produce ATP through fermentation (Schleifer and Kilpper-Bälz, 1987; Axelsson, 2004).

*L. delbrueckii* ssp. *bulgaricus* is commonly used alongside *S. thermophilus* as a starter culture for making yogurt. During fermentation, growth of *S. thermophilus* is fastest in the early stage, but as the pH drops below 5.5, its growth slows and *L. delbrueckii* ssp. *bulgaricus* tends to predominate (Adams and Moss, 2000). Both species produce lactic acid. It gives yogurt its sour flavor; the resulting decrease in pH partially coagulates the milk proteins and thickens the yogurt. By the end of fermentation, the yogurt has a titratable acidity (TA) of 0.90%–0.95%, expressed as lactic acid, and with the populations of the two species in excess of $10^8$ cfu/mL. The two species work in synergy with one another, but are not completely interdependent (Sieuwerts et al., 2010). Both LAB species can ferment milk on their own, but they grow faster and produce acids faster when together. It has been reported that the growth of *S. thermophilus* in milk is limited by the availability of peptides and free amino acids, which are typically present in relatively low or rate-limiting concentrations (Mäyrä-Mäkinen and Bigret, 2004).

*L. delbrueckii* ssp. *bulgaricus* is slightly proteolytic and liberates small amounts of peptides and amino acids, particularly valine, which stimulates the growth of *S. thermophilus*. In return *S. thermophilus* produces formate, pyruvate, and carbon dioxide, all of which stimulate the growth of *L. delbrueckii* ssp. *bulgaricus* (Mäyrä-Mäkinen and Bigret, 2004). While fermenting milk, *L. delbrueckii* ssp. *bulgaricus* produces acetaldehyde, one of the main yogurt aroma components; this compound is an oxidation product that has an ethereal, fresh, and pungent smell (Cheng, 2010). In addition to lactic acid and acetaldehyde, the compounds diacetyl, 2-butanone, acetone, and acetoin also contribute to the typical aroma and flavor of yogurt. Diacetyl smells like butter, cream, and vanilla; 2-butanone has a varnish-like, sweet and fruity note; acetone has a sweet and fruity aroma; while acetoin is buttery in character (Cheng, 2010; Rincon-Delgadillo et al., 2011). For an overview of the ~100 volatile compounds associated with yogurt, readers are referred to the comprehensive reviews by Cheng (2010) and Routray and Mishra (2011).

The roles of LAB in yogurt fermentation are unique; they not only give yogurt a special taste (flavor), but inhibit other microorganisms by competing for nutrients, rapidly producing lactic acid and
other acids and generating antimicrobial compounds such as acetaldehyde, diacetyl, hydrogen peroxide, and bacteriocins. Bacteriocins are proteinaceous compounds produced by some LAB that inhibit the growth of similar or closely related bacterial strains. It is these characteristics that allow LAB to improve the quality and safety of yogurt products.

12.1.3 THE OUTLINE OF YOGURT PRODUCTION

The first prerequisite of any milk used in yogurt fermentation is that it should be free from antimicrobials. The inhibition of the starter culture by an antibiotic would not only result in economic losses, but also potentially allow pathogens to grow. In commercial practice, homogenized milk is almost exclusively used for yogurt production to prevent milk fat separation and improve product stability. In addition, it is usual to supplement the solids content of the milk to also enhance the final product texture. This is done by increasing the milk SNF content, from 8.5% to 11%–15%, by the addition of skim milk or whole milk powder, depending on whether a conventional or low-fat product is to be made (Adams and Moss, 2000). The properties of a yogurt may also be improved and stabilized by the addition of small amounts of natural or modified gums; these additives bind water and thicken the product.

Milk is heated to 85°C for about 30 min prior to adding the starter culture; this pasteurizes the milk so that the starter culture can grow without competition from other microorganisms. The heating process also expels oxygen to create a microaerophilic environment (Adams and Moss, 2000). The production of small molecular weight sulfur-containing compounds such as $\text{H}_3\text{C-S-CH}_3$ has been reported in milk because of heat treatment, and the reactive sulfhydryl groups increase lactase activity (Jiménez-Guzmán et al., 2002). Heating also promotes interaction between whey or serum proteins and casein, which increase the yogurt viscosity, stabilize the gel, and limit syneresis (separation of whey) (Soukoulis et al., 2007).

The heat-treated milk is cooled to 45°C, which is the near optimal fermentation temperature for both $S. \text{thermophilus}$ and $L. \text{delbrueckii}$ ssp. bulgaricus. The starter culture is then added at a level of about 2% (v/v) to give an initial LAB population of $10^6$–$10^7$ cfu/mL, comprised of approximately equal populations of both organisms.

Fermentation can be conducted in retail packs to produce a firm and continuous coagulum, which is known as a set yogurt, or in bulk tanks to produce a stirred yogurt when other ingredients are mixed in prior to packing.

Fermentation takes place at 40–43°C for about 4–6 h depending on the starter culture used and the initial pH of the milk (6.3–6.5). The lactic acid produced by the LAB helps destabilize the complex of casein micelles and denatured whey proteins by solubilizing the calcium and phosphate ions; when the pH reaches 4.6–4.7, the isoelectric point of the casein, the micelles aggregate to produce a continuous gel in which all the components are entrapped with little or no syneresis (Schorsch et al., 2001; Soukoulis et al., 2007).

When fermentation is complete, the yogurt is cooled to 15–20°C before the addition of sugar, flavor, and/or fruit(s) prior to packaging. It is then cooled further to 4–5°C and can be stored for up to approximately 4 weeks. The acidity may continue to increase slowly during storage because of the low level of LAB activity. Because of the high acidity and low pH (3.8–4.2) of yogurt, many other microorganisms will not grow and/or survive during storage.
12.2 MICROBIOLOGY OF LACTIC ACID BACTERIA IN CARROT JUICE AND MILK YOGURTS

12.2.1 CARROTS

Carrot (Daucus carota L.), pumpkin, and spinach rank highest on the list of commonly consumed anti-oxidant vegetables, with a high β-carotene content as well as other health-supporting nutrients. The β-carotene in carrots (6.9–15.8 mg carotenoids/100 g) provides a strong antioxidant that quenches free radicals and protects cells against oxidative damage (Bandyopadhyay et al., 2007).

While considerable carrot research has focused on carotenoids and their antioxidant benefits (Sharma et al., 2012), recent research has identified another category of phytonutrients called poly-acetylenes, with beneficial effects (Christensen, 2013; El-Houri et al., 2015). The most important poly-acetylenes include falcarinol and falcarindiol; these compounds can inhibit the growth of cancer cells, especially when they are present in their reduced (vs oxidized) form (Christensen, 2013). These findings suggest that the carotenoid-rich carrots not only help prevent oxidative damage inside our body, but may also prevent oxidative damage to the carrot polyacetylenes themselves, thereby maximizing health benefits.

In addition, carrot (juice) is regarded as a prebiotic vegetable that stimulates the growth or activity of bacteria in the digestive system, which are beneficial to the health of the human body (Slavin, 2013).

Other current research has classified fruits and vegetables into four color categories: green, orange/yellow, red/purple, and white (Pennington and Fisher, 2010). Fruits and vegetables with the deepest shades of orange/yellow have been shown to offer the greatest protection against cardiovascular disease (CVD), as exemplified by carrots. Oude Griep et al. (2011) determined that research participants who had the lowest carrot intake had the least CVD risk reduction; those who ate at least 25 g of carrots had a higher CVD risk reduction, while those who ate more than 50 g had a significantly greater CVD risk reduction.

12.2.2 CASE STUDY OF CARROT JUICE YOGURT

12.2.2.1 Introduction and Research Objectives

Lactic acid fermentation technology is a low-cost and effective means of processing and preserving foods with added value. Food and beverage markets are facing an increasing demand for all natural, functional, and minimally processed foods (Annunziata and Vecchio, 2011; Shibly and Mishra, 2013).

While most new yogurts are formulated by the addition of flavorants that are added after fermentation, this research sought to develop a yogurt with the flavorants (carrot juice) added prior to fermentation. Research at the Kentville Research and Development Centre (KRDC), NS, Canada, focused on developing an all-natural unsweetened yogurt made from carrot juice and milk (Cliff et al., 2013). The new carrot juice–milk yogurts combined the beneficial properties of LAB and the nutritional components from milk and carrot juice. Cliff et al. (2013) characterized the sensory characteristics (color, aroma, flavor/taste, and texture/mouth feel) of the carrot juice–milk yogurts, as well as determined their consumer acceptability, using consumers in Wolfville, NS and Vancouver, BC. While consumers in NS were slightly more accepting of the unsweetened carrot juice yogurts than those in BC, further research was deemed necessary to establish an appropriate sweetener to improve the yogurt’s overall acceptability.

The objectives of this research were to track the fate of the LAB and the composition of the yogurts (plain, carrot juice) during 4 weeks of storage at three temperatures. Storage temperatures were selected...
to represent refrigerator (4°C), cooler (10°C), and ambient (20°C) temperatures, to simulate temperatures associated with use and abuse/misuse conditions. Viability assays and fluorescent techniques were used to determine live/dead microorganisms, while compositional analyses tracked the pH and lactic acid concentration.

### 12.2.2.2 Materials and Methods
#### 12.2.2.2.1 Yogurt Production
The starter culture consisted of *L. delbrueckii* ssp. *bulgaricus*, *S. thermophilus*, and *L. acidophilus* (LYO-San Inc., Lachute, QC, Canada).

Plain yogurt was prepared using 2% skimmed homogenized milk, starter culture, skim milk powder to give 13.77% milk SNF, and 0.35% (w/v) gelatin. Carrot juice yogurt was made with the identical ingredients as the plain yogurt, but with the addition of 33% carrot juice (Picture 12.1). Briefly, skim milk powder and unflavored gelatin were added to the 2% skimmed pasteurized milk, or milk containing carrot juice, and heated at 80°C for 30 min. The mixtures were cooled to 45°C, and starter cultures were added, mixed, and dispensed into 150 mL yogurt cups. Yogurts were fermented at 40°C for 4 h, and stored at 4, 10, or 20°C. Yogurt samples were analyzed at 0, 1, 2, 3, and 4 weeks.

#### 12.2.2.2.2 LAB Plate Count
A yogurt sample (1 mL) was used to prepare serial dilutions, which were then surface plated on duplicate plates of both deMan, Rogosa, Sharpe agar and *S. thermophilus* agar using a Whitley automatic spiral plater (Don Whitley Scientific Limited, West, Yorkshire, UK). The plates were incubated anaerobically at 42°C for 48 h and the colonies of *Lactobacillus* spp. and *S. thermophilus* were counted using an Acolyte colony counter (a division of the Synoptics Group, Cambridge, UK). LAB populations were expressed as log cfu/mL.

#### 12.2.2.2.3 Epifluorescence Microscopy
Epifluorescence microscopy was used to determine the viability of LAB in yogurt. Using a LIVE/DEAD BacLight Bacterial Viability kit (L7012, Invitrogen, Burlington, ON, Canada), changes in proportions of live (green fluorescence) and dead (red fluorescence) bacteria were measured to provide an

![Picture 12.1](image)

Plain and carrot juice yogurts.
indicator of the physiological status of the cells (Maukonen et al., 2006). Because of a high population of LAB in yogurts, samples were diluted to approximately $10^7$cells/mL prior to staining. In brief, equal volumes of component A and component B in kit L7012 were combined in a microfuge tube and mixed thoroughly; $3 \mu L$ of the dye mixture was added for each milliliter of the LAB suspension and incubated at room temperature in the dark for 15 min. A $5 \mu L$ sample of the stained LAB suspension was then taken for enumeration of live and dead bacterial cells. The enumeration of fluorescent cells was performed using a Nikon epifluorescent microscope (Nikon Eclipse 80i, Nikon Canada Inc., Mississauga, ON, Canada) equipped with a 100W mercury arc lamp and through a 40×/0.75 objective lens and a Neubauer-improved hemocytometer (Hausser Scientific, Horsham, PA, USA). LAB populations were expressed as log cells/mL.

12.2.2.2.4 Compositional Determinations of pH and Lactic Acid Concentration
The pH of the yogurt was determined using an AR15 pH meter (Fisher Scientific, Montreal, QC, Canada). TA was determined using a PC-Titrate System (Man-Tech Associates Inc., Guelph, ON, Canada). A 20mL yogurt sample was titrated to an endpoint of pH 8.5 using 0.5N NaOH, and calculated as g lactic acid equivalents/100mL yogurt and reported as percent (%) lactic acid.

12.2.2.2.5 Statistical Analysis
The experiment was conducted using a split-plot design. The two yogurt types (plain or carrot juice yogurt) and three storage temperatures (4, 10, or 20°C) were completely randomized to the main plots and storage times (0, 1, 2, 3, or 4 weeks) randomized to the split-plot. The entire experiment was replicated three times, with the exception that only two replications of the epifluorescence microscopy counts were performed.

A three-factor analysis of variance (ANOVA) was conducted including the main effects of yogurt type (Y), storage temperature (T), storage time (S) as well as all two- (Y × S, T × S) and three-factor (Y × T × S) interactions. The exact probabilities ($P$) of the $F$ values were reported. Mean values were compared using the SEM, rather than conducting mean separation tests, as suggested by Chew (1976) and Bryan-Jones and Finney (1983). In general, means that differed by three times the SEM were significantly different at $P \leq .05$.

A principal component analysis (PCA) was conducted on the microbiological and chemical determinations [pH, TA, LAB anaerobic plate count, live LAB (cells/mL), and dead LAB (cells/mL)] using the correlation matrix. A biplot was created using the PCA scores and factor loading to compare the similarities of the yogurts and understand the relationship between the microbiological and chemical determinations. All statistical analyses were conducted using GenStat software (VSN International, 2011) for Windows.

12.2.2.3 Results and Discussion
12.2.2.3.1 LAB Anaerobic Plate Counts at Different Temperatures and Storage Times
ANOVA revealed that yogurt type did not have a significant effect ($P = .16$) on LAB anaerobic plate counts (Table 12.1). However, there was a significant effect for the interaction of storage temperature and storage time ($T \times S$) ($P < .001$), as well as for the interaction among yogurt type, storage temperature, and storage time ($Y \times T \times S$) ($P = .002$) (SEM = 0.152). Mean LAB anaerobic plate counts (Table 12.1) that differ by more than 0.456 (i.e., three times SEM) were significantly different at $P \leq .05$. 
After 4 weeks of storage at 4, 10, and 20°C, the LAB anaerobic plate counts in plain yogurt (control) were 8.70, 8.09, and 4.94 log cfu/mL, respectively, while those in carrot juice yogurts were 8.31, 7.25, and 5.39 log cfu/mL, respectively. These decreasing plate counts with increasing temperature reflect the loss of viability of the LAB over time, with utilization of substrates and production of by-products. At 4°C, the LAB anaerobic plate counts were all greater than 8.30 log cfu/mL for all samples for up to 4 weeks of storage. Thus all yogurts would be consistent with the guidelines that yogurts should contain more than $10^8$ cfu/g (i.e., log 8) live cells (National Yogurt Association, 2013). A dramatic decrease in LAB anaerobic plate counts (live LAB) was observed for yogurts (plain, carrot juice) stored for 4 weeks at 10°C and more than 2 weeks at 20°C (Table 12.1).

Since carrot juice was pasteurized prior to mixing with the milk, there was no fungal contamination in the yogurts after storage (data not shown). This is consistent with the work of Salwa et al. (2004) who reported that the addition of carrot juice was advantageous to the yogurt fermentations because of the presence of aflatoxin M1, which acted as an antibacterial and antifungal agent.

**12.2.2.2 pH and Lactic Acid Concentrations in Yogurts**

ANOVA revealed that yogurt type, storage temperatures, and storage times all had significant effects ($P<.001$) on the pH of the yogurts (Table 12.2). There was a significant interaction between storage temperature and storage time ($T \times S$) ($P<.001$), but not for the interaction among yogurt type, storage temperature, and storage time ($T \times S \times Y$) ($P>.05$).
temperature, and storage time (Y × T × S) \( (P = .552) \) (SEM = 0.02). Mean pH values (Table 12.2) that differ by more than 0.060 (i.e., three times the SEM) are significantly different at \( P \leq .05 \).

In general, the pH of the carrot juice yogurts was slightly higher compared to the plain yogurts, initially (week 0), as well as throughout the storage period (weeks 1–4) (Table 12.2). After 4 weeks of storage at 4°C, pH levels remained unchanged at 3.94 and 4.08 in plain and carrot juice yogurts, respectively. This suggested that the fermentations were inactive.

In contrast, there was a drop [negative change (\( \Delta \))] in pH over the 4 weeks at 10°C for plain (3.95–3.79; \( \Delta = -0.16 \)) and carrot juice (3.97–3.79; \( \Delta = -0.18 \)) yogurts. The difference between the yogurt types (plain, carrot juice) was nonsignificant. A larger drop in pH was observed over the 4 weeks at 20°C for the plain (3.88–3.59; \( \Delta = -0.29 \)) and carrot juice (3.91–3.69; \( \Delta = -0.22 \)) yogurts. In this case, there was a difference between the yogurt types, with a greater pH change (drop) for the plain yogurt compared to the carrot juice yogurt. This was attributed to greater loss of viability of the LAB in the carrot juice formulations, especially with higher storage temperatures and longer storage times—consistent with the LAB anaerobic plate counts observed in Table 12.1.

ANOVA also revealed that yogurt type, storage temperatures, and storage times all had significant effects \( (P < .001) \) on the TA (% lactic acid) of the yogurts (Table 12.3). There was a significant interaction among yogurt type, storage temperature, and storage time (Y × T × S) at \( P = .016 \) (SEM = 0.045). Mean lactic acid concentrations (Table 12.3) that differed by more than 0.135 (i.e., three times the SEM) were significantly different at \( P \leq .05 \).

In general, the TA of the carrot juice yogurts was slightly lower than those of the plain yogurts initially (week 0), as well as throughout the storage period (weeks 1–4) (Table 12.3). This was in part attributed to the incorporation of carrot juice with a different nutrient composition in the formulation to that of plain yogurt, as well as consistent with the higher pH values observed in Table 12.2 for the carrot juice yogurts.

After 4 weeks of storage at 4°C, lactic acid concentrations remained unchanged at 1.43% and 1.21% in plain and carrot juice yogurts, respectively. This suggested that the fermentations were inactive at 4°C. In contrast, there was an increase [positive change (\( \Delta \))] in lactic acid concentrations over the 4 weeks at 10°C for plain (1.42–1.58; \( \Delta = +0.16 \)) and carrot juice (1.36–1.57; \( \Delta = +0.21 \)) yogurts. The difference between the yogurt types (plain, carrot juice) was nonsignificant. A larger increase in the lactic acid concentration was observed over the 4 weeks at 20°C than at 10°C for the plain (1.48–1.83; \( \Delta = +0.35 \)) and carrot juice (1.43–1.76; \( \Delta = +0.33 \)) yogurts because of the activity (viability) of the LAB populations (Table 12.1). These patterns were consistent with the changes in pH observed in Table 12.2, although in the opposite

<table>
<thead>
<tr>
<th>Yogurt Type</th>
<th>Storage Temperature (°C)</th>
<th>Week 0</th>
<th>Week 1</th>
<th>Week 2</th>
<th>Week 3</th>
<th>Week 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plain yogurt</td>
<td>4</td>
<td>1.38</td>
<td>1.32</td>
<td>1.35</td>
<td>1.42</td>
<td>1.43</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>1.42</td>
<td>1.58</td>
<td>1.55</td>
<td>1.61</td>
<td>1.58</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>1.48</td>
<td>1.66</td>
<td>1.86</td>
<td>1.87</td>
<td>1.83</td>
</tr>
<tr>
<td>Carrot juice yogurt</td>
<td>4</td>
<td>1.23</td>
<td>1.31</td>
<td>1.24</td>
<td>1.24</td>
<td>1.21</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>1.36</td>
<td>1.34</td>
<td>1.52</td>
<td>1.58</td>
<td>1.57</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>1.43</td>
<td>1.76</td>
<td>1.77</td>
<td>1.77</td>
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</tr>
</tbody>
</table>
direction because of the inverse (nonlinear) relations between pH and acidity. Since Bodyfelt et al. (1988) identify that a TA above 1.25% and a pH below 3.8 are considered too “high acid,” it is recommended that the carrot juice yogurts be stored at the recommended temperature (4°C) without temperature fluctuations or temperature abuse.

12.2.2.3.3 Principal Component Analysis of Yogurts
PCA (Fig. 12.1) of the scores explained 70.0% and 19.0% of the variation along PC 1 and PC 2, respectively; an additional 8.0% was account for along PC 3 (data not shown). PC 1 was more heavily loaded in the negative (−) direction with “dead LAB (cells/mL)” and “titratable acidity (% lactic acid)” and in the positive (+) direction with “LAB anaerobic plate count” and “pH,” while “live LAB (cells/mL)” were most heavily loaded on negative PC 2 (Fig. 12.1).

PC 1 can be thought of as a continuum from “high pH, high LAB anaerobic plate count” to “high TA, high dead LAB,” moving from right to left in the biplot (Fig. 12.1). While PC 2, heavily loaded with “live LAB,” can be thought of as a continuum of “high to low live LAB,” moving from bottom to top in the biplot (Fig. 12.1).

The most striking feature of the biplot was the delineation of the yogurts by storage temperature. Yogurts stored at 4, 10, and 20°C were positioned to the right, centrally, and to the left, respectively. Yogurts stored at 4°C, located on the right, had the highest pH and LAB anaerobic plate counts, and lowest TA and dead LAB. Yogurts stored at 10°C, positioned in the center, had intermediate levels of these characteristics (pH, anaerobic plate count, TA, dead LAB), while yogurts stored at 20°C, located on the left, had the highest TA and dead LAB and lowest pH and LAB anaerobic plate counts—with the exception of the freshly prepared yogurts that had not been stored at each of the three temperatures (4°C-0 week, 10°C-0 week, and 20°C-0 week). These yogurts were positioned similarly, in the upper right, and as expected had similar traits (high pH and LAB anaerobic plate counts).

FIGURE 12.1
Principal component analysis of yogurt data where 4, 10, and 20 indicate the storage temperature in °C, and 0, 1, 2, 3, and 4 indicate the storage time in weeks. Plain and carrot juice yogurts are indicated in black and red (gray in print versions) fonts, respectively. Yogurt stored at the three storage temperatures has been encircled to aid visualization.
Yogurts stored at 4°C formed the tightest grouping in Fig. 12.1. These characteristics were very similar independent of storage time (4°C-0 week, 4°C-1 week, 4°C-2 weeks, 4°C-3 weeks, and 4°C-4 weeks), with high pH and high LAB anaerobic plate counts. This is consistent with the fact that cultures would have had very little activity at 4°C. In contrast, yogurts stored at 10°C formed a looser grouping in the center of the biplot (Fig. 12.1). Within this grouping there was a trend for yogurts at the early storage times (10°C-1 week, 10°C-2 weeks) to be positioned to the right of those at the later storage times (10°C-3 weeks, 10°C-4 weeks), suggesting that these yogurts had lower pH and higher TA with longer storage. This pattern was also observed for the yogurts stored at 20°C. These yogurts formed an extremely loose grouping, positioned on the upper and lower left-hand side of the biplot (Fig. 12.1). Within this grouping, there was a trend for yogurts with increasing storage times to be located to the left of each other, with each additional week of storage (20°C-1 week, 20°C-2 weeks, 20°C-3 weeks, and 20°C-4 weeks). This reflected that both plain and carrot juice yogurts had progressively decreasing pH and increasing TA with duration of storage.

However, another pattern was also evident in Fig. 12.1, within the 10 and 20°C groupings. In general, carrot juice yogurts were positioned higher in the plane than plain yogurts for each of the storage times, separated along PC 2. This reflected that the plain yogurts had higher live counts than their carrot juice yogurt counterparts, especially after 3 and 4 weeks of storage at 10°C (10°C-3 weeks, 10°C-4 weeks) or after 2, 3, or 4 weeks of storage at 20°C (20°C-2 weeks, 20°C-3 weeks, and 20°C-4 weeks). Conversely, the carrot juice yogurts had higher live counts when stored at 4°C for up to 4 weeks, or at 10°C for up to 2 weeks. Warming the carrot juice yogurts to approximately room temperature, particularly for extended periods of time (2–4 weeks), was detrimental to their nutritive and sensory properties.

In general, small increases in temperature (~10°C) enhanced viability of the cultures (i.e., live counts), with only modest increases in TA. However, higher storage temperatures (20°C) resulted in no further increase in viability, but a substantial increase in TA. If high storage temperatures (20°C) were maintained for an extended period (2–4 weeks), viability of the LAB cultures dropped off substantially with each additional week (Fig. 12.1). This pattern was more pronounced for the carrot juice yogurts than the plain yogurts. This drop in viability (death) was attributed to the reduction of substrate (lactose), but also to the increase in concentration of the end products (lactic acid).

12.3 CONCLUSION

The new carrot juice yogurts were developed by cofermenting milk and carrot juice with the addition of LAB starter culture. Pasteurization of the carrot juice prior to incorporating it into the milk, along with good sanitation practices, ensured that there was no fungal contamination in the yogurts over the 4 weeks of storage period. The unique combination of milk and carrot juice retained its yogurt characteristics and orange color throughout fermentation and storage (Salwa et al., 2004). Since the acidity of carrot juice yogurts increased with storage temperature and time, the carrot juice yogurts should be stored at the recommended temperature (4°C) and consumed shortly after fermentation (1–3 weeks). This would not only avoid increases in acidity, but also ensure maximum viability of the LAB (>10⁸ cfu/g). This research along with previous research (Cliff et al., 2013), illustrating color and texture acceptability, documented the technical possibility of producing yogurts with 33% carrot juice.
Based on this technology, a range of vegetable yogurts might be produced, not just with carrot, but potentially with beet, sweet potato, squash, or sweet red pepper.

**12.3.1 SENSORY ACCEPTABILITY OF YOGURTS**

Consumers are seeking new foods and/or supplements to enhance their nutrition and health (Hekmat and Reid, 2006), acquire the benefits associated with the ingestion of live microorganisms (Shortt, 1999; Giraffa et al., 2010), and prevent disease. As a result, there has been a proliferation of new dairy products in the marketplace including fermented milk products, yogurt drinks, pre- and probiotic yogurts, low-fat dairy alternatives, and Greek yogurts, to name a few.

Consumers realize that to eat healthily they, and their families, should increase their consumption of vegetables (US Department of Health and Human Services and US Department of Agriculture, 2015). However, vegetable yogurts are currently not available in the North American marketplace.

With this in mind, Cliff et al. (2013) made progress toward developing a carrot juice yogurt that would offer the consumer the nutritional benefits of a fermented milk product, along with the high β-carotene content of carrots. Cliff et al. (2013) prepared carrot juice yogurts with different levels of carrot juice (8%, 16%, 24%, and 32%), two firmness levels (regular, 45 g/L milk solids; firm, 90 g/L milk solids), and two starter cultures. They characterized these yogurts using a combination of descriptive analysis and consumer research. Using these tools in tandem, they were able to characterize the visual, aroma, taste, and textural attributes, as well as understand the sensory attributes responsible for consumer acceptance of the new yogurt.

Incorporating the “voice of the consumer” at the early stages of product development has been demonstrated to be important for achieving success of a new product in the marketplace (van Kleef et al., 2005). Cliff et al. (2013) showed that the use of carrot juice in the yogurt formulation significantly influenced the sensory and hedonic scores. The overall hedonic scores decreased with increasing carrot juice concentration, suggesting that the carrot juice directly or indirectly introduced constituents that were less than ideal. Anecdotal comments collected from consumers suggested that the low hedonic ratings were partially attributed to vegetative aromas/flavors and sour/lactic notes—especially for Asian consumers who had lower acceptability scores for the carrot juice yogurts than non-Asian consumers.

Such remarks were consistent with research by Harper et al. (1991) who suggested that plain yogurt was simply too sour for many consumers, and Prescott et al. (1992) who reported that Asian consumers scored acid (sour) foods lower than their non-Asian counterparts. In this research, carrot juice yogurts were indeed more acidic (1.23%) than commercially available yogurts (0.9%–1.2%) (Bodyfelt et al., 1988). Therefore a lower acidity would likely improve the overall acceptability of these new yogurts, if commercialized.

Consumers also commented (Cliff et al., 2013) that the carrot juice yogurts would likely be acceptable if sweetened. This was consistent with Vickers et al. (2001) who identified that lower than optimal sweetness levels for yogurt had detrimental effects on taste ratings. Consumers suggested that a small amount of sugar, honey, or stevia added to the carrot juice yogurts would have made them very acceptable. Parents were particularly impressed by the fact that a 250 mL yogurt serving would contribute almost a full serving (64%) of vegetables, according to the Canadian Food Guide (Health Canada, 2011).
As such, the carrot juice yogurt prototypes developed by Cliff et al. (2013) would need further improvement prior to release into the marketplace, with careful selection of a cultivar with minimal vegetative notes. New formulations would also need to pay close attention to consumers’ preferences for organic and/or sustainable ingredients that might influence success in the marketplace. A full appreciation of consumers’ acceptance for a new product such as vegetable yogurts would need to be captured using a combination of tools, such as focus groups (Thompson et al., 2007), preference maps (Tu et al., 2010), neophobia scales (Henriques et al., 2009), and possibly emotion measurements (Mojet et al., 2015).

12.4 FUTURE RESEARCH

Yogurts provide a unique combination of nutritional and therapeutic benefits, from the milk, other ingredients (fruits, vegetable juices), and live LAB cultures. Their role as a functional food in a healthy diet is undisputed (Annunziata and Vecchio, 2011). Individuals who consume yogurt can reduce their risk of chronic diseases (hypertension, CVDs, diabetes, and some cancers) as well as maintain intestinal regularity (Pei et al., 2016).

Along with enhanced nutritional and healthy benefits, food companies demand improved LAB starter cultures containing multiple LAB strains that provide reliable fermentations, are resilient to bacteriophages, and produce consistent flavor and texture properties (Donovan and Shamir, 2014; Pei et al., 2016). However, much remains to be understood about the interaction and control of multiple strains within a starter culture (Mäyrä-Mäkinen and Bigret, 2004).

Fermented dairy products also contain probiotic LAB, such as L. acidophilus and Bifidobacterium spp. These probiotic effects are known to be strain dependent, so it is important to investigate the viability of the probiotic organisms within the product, during the shelf-life, and in the gastrointestinal tract. Yet, the mechanism of action responsible for the health benefits remains to be completely understood (Shortt, 1999; Slavin, 2013).

It is possible that cofermentation of milk with carrot juice (Cliff et al., 2013) could alter the chemical structure of carotenes. Much remains to be understood about the bioactivity and bioavailability of carotenes and other functional compounds after fermentation and prior to consumption.

Modern consumers require that their foods be not just nutritious, but organic and sustainable too. While organic yogurts are available in the marketplace, sustainable yogurts are not. Brown and Chambers (2015) have been successful in developing three sustainable yogurt prototypes that required less energy for production than conventional yogurts.

The sensory characteristics associated with the ideal profiles of French consumers were dominated by differences in textural characteristics (thickness, granularity, flowing texture, stickiness) (Masson et al., 2016). The consistency of yogurt can vary by a factor of 2–15 times (Sodini et al., 2004), from a thin drinkable liquid, to a thick custard-like consistency. Despite considerable research, little is known about the contribution of protein and fat to yogurt structure, as well as the optimal whey protein and casein concentrations required to produce a desired texture (Sodini et al., 2004).

Research by Saint-Eve et al. (2016) has quantified just how much sugar French consumers add to their yogurt prior to consumption. They identified three groups of consumers, “low,” “medium,” and “heavy” sugar users, who added 6.1, 11.4, and 19.9 g of a sweetener (per 125 g yogurt), respectively. Surprisingly, none of the consumers’ ideal sweetness levels corresponded to the amount that is
currently added to presweetened yogurt (12.6 g sugar/125 g yogurt) in the marketplace, suggesting that commercial yogurts have a less than optimal composition.

Consumer preference of yogurt is also driven by nonsensory characteristics such as brand, price, and health claims (Ares et al., 2008), as well as environmental claims (“organic,” “sustainable,” “non-GMO”) and dietary claims (“low fat,” “low calorie”) (Niedziela et al., 2016). The contribution of these factors is important to success in the marketplace and needs to be considered when developing new yogurts and/or refining the characteristics of yogurts for existing markets.

Pohjanheimo and Sandell (2009) identified that the characteristics of yogurts that were preferred by consumers who valued “natural content, ethical concern, and health” were not the same as those preferred by consumers who valued yogurt for its “convenience, price, mood, and familiarity.”

Looking ahead, the demand for yogurt would be expected to increase as consumers place importance on healthy eating and wellbeing, maintaining their weight, using food as preventive medicine, and caring about agricultural sustainability. As such, this suggests the yogurt market will likely remain strong and the food industry will have the opportunity to develop new products to meet the needs of consumers.

In closing, there is much research to be done in the product category to understand the improved LAB starter cultures, the probiotic strains, the mechanisms of protein/fat interactions in the formation of texture, the bioactivity and bioavailability of the functional compounds, the constituents responsible for creaminess, the ideal sweetness for all subgroups of consumers including children, and the role of the nonsensory factors responsible for yogurt choices, to name a few.

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13.1 INTRODUCTION

The human gastrointestinal (GI) tract harbors complex microbial communities, comprising bacteria, Archaea, fungi, protozoa, and viruses. This microbial ecosystem, or intestinal microbiota, is principally confined or passing through the small intestine (mainly populated by facultative anaerobic and aerobic bacteria) and colon (mainly populated by anaerobic bacteria). The combined genomes (5 million genes) of these microbes, and the surrounding environmental conditions, are known as the intestinal microbiome (Holmes et al., 2012; Marchesi and Ravel, 2015; Weinstock, 2012). The bacterial content increases distally, from $10^0$–$10^3$ bacteria/mL of gastric contents in the stomach, progressing to $10^5$–$10^8$ bacteria/g of jejunal and ileal contents and up to $10^{11}$–$10^{12}$ bacteria/g (dry weight) in the colon, which is the most densely colonized area of the GI tract (Gerritsen et al., 2011).

The intestinal microbiome has coevolved with the human host to perform a number of physiological, nutritional, and immunological functions, including helping to degrade food particles, breaking down xenobiotics and carcinogens, producing short-chain fatty acids (SCFA) and essential vitamins, and providing protection against bacterial pathogens. The different bacterial groups can also stimulate the development of the mucosal and humoral immune system and the differentiation and proliferation of the host’s intestinal epithelial cells that regulate the intestinal homeostasis (Hooper et al., 2012; Sommer and Bäckhed, 2013, 2016).

In healthy individuals, the diversity and composition of the intestinal microbial populations interact with the innate and adaptive immune systems to maintain intestinal homeostasis. Fluctuations of the balance between the commensal organisms with beneficial potential (symbionts) and commensal microorganisms with pathogenic potential (pathobionts) caused by environmental or nutritional factors lead to a disruption of this homeostasis (Round and Mazmanian, 2009). An alteration of the intestinal environment caused by oxidative and inflammatory stress, associated with a homeostatic imbalance, partly explains the link between the dysbiosis defined by a change in the structural or functional configurations of the gut microbiome and the development of metabolic diseases and disorders that affect the human digestive tract. Indeed, a disruption of immune tolerance to commensal bacteria and a dysregulation of the intestinal microbiota balance (“dysbiosis”) may result in gut inflammation, epithelial dysfunction, and enhanced mucosal permeability (Marchesi et al., 2016).

Various disease states are associated with quantitative and qualitative alterations of the intestinal microbiota. These diseases can include enteric infections (Clostridium difficile, Salmonella spp., and Campylobacter jejuni), inflammatory bowel diseases (Crohn’s disease and ulcerative colitis), small intestinal bacterial overgrowth, cardiovascular diseases, nonalcoholic fatty liver disease, and colorectal...
cancer, but also GI disorders (irritable bowel syndrome), obesity, and related metabolic disorders (insulin resistance and type 2 diabetes) (Aziz et al., 2013).

Other factors such as antibiotic administration or high-fat diet disturb the intestinal microbiome resulting in decreased bacterial diversity and richness. In addition, the long-term use of antibiotics during infancy can have adverse effects on the microbiome resulting in an increasing incidence of atopic and autoimmune diseases and obesity (Lozupone et al., 2012).

The use of prebiotics, probiotics, and fecal transplantation therapy has been proposed to alleviate these disturbances of fecal microbiota by stabilizing the intestinal bacterial communities and minimizing potential alterations in microbial community structures (Mondot et al., 2013). Probiotics are defined as “live microorganisms which, when administered in adequate amounts, confer a health benefit on the host” (Hill et al., 2014). Commercial probiotic strains can be consumed as supplements or incorporated into fermented milk or yogurt products. This chapter presents the current knowledge about the possible impact of probiotic yogurts on the modulation of fecal microbiota.

13.2 THE FECAL MICROBIOTA

The development of the intestinal microbiota is a complex process that is not limited to a simple ecological succession of microbial populations. Several internal and external factors such as host genetics, mode of delivery (vaginal birth vs. cesarean section), type of diet (breastfeeding vs. formula feeding), gestational age, infant hospitalization, and antibiotic use, as well as host–microorganism interactions, influence postnatal colonization (Collado et al., 2012, 2015). Microbial colonization of the intestinal tract takes place in four successive phases (Rodríguez et al., 2015). The first phase occurs during and after birth, and is characterized by a large fluctuation of opportunistic bacterial species that are able to colonize the GI tract. Phase 2 is primarily influenced by breastfeeding or milk-based formulas. Phase 3 corresponds to that of food diversification following weaning and introduction of solid foods. Finally, at the end of the first year (Phase 4), the composition of the gut microbiota, individually distinct, converges to that of an adult, and fully resembles the characteristic microbiota (highly diversified and dominated by Firmicutes and Bacteroidetes) of adults after 2.5 years. This sequential colonization plays a key role in the development of a robust and diverse intestinal microbiota, demonstrating resilience and optimal functionality by facilitating the formation of a physical and immunological barrier between the environment and the host.

13.3 MICROBIAL COLONIZATION

Each ecological niche of the GI tract is likely to become occupied by well-adapted microorganisms, which are often passed on by the mother. For a long time, the fetal environment was assumed to be sterile and that microbial colonization began during birth and shortly thereafter. This microbial colonization only came from the maternal vaginal microbiota. The intestinal microbiota gradually becomes complex and diverse depending of the mode of feeding during the first months. The latest findings challenge this hypothesis. The placenta, amniotic fluid, fetal membranes, and cord blood harbor microorganisms. Bacteria in the intrauterine environment, as those detected in the amniotic fluid, could be associated with the prenatal colonization meconium (the first stool of the neonate) (Mueller et al., 2015).
In Phase 1, the first colonizers in the neonatal gut are generally aerobic and facultative anaerobic such as Enterobacteriaceae, enterococci, streptococci, and staphylococci. During the first days, they consume oxygen during their growth and create a suitable environment for the colonization of strict anaerobic bacteria such as *Bifidobacterium*, *Bacteroides*, and *Clostridium* spp. The neonatal microbiota is highly dynamic and unstable, and is characterized by low diversity and a relative dominance of *Proteobacteria*. The presence of *Lactobacillus* was also observed in neonates within the first 48 h of life. The abundance of aerobic and facultative bacteria rapidly decreases under the influence of oxygen depletion, substrate competition, and accumulation of toxic metabolites such as organic acids produced by lactobacilli and bifidobacteria (Rodríguez et al., 2015).

Marked differences are observed between the intestinal microbiota of babies born by caesarean section and those born naturally. The microbiota of the first infant group is dominated by bacterial communities similar to those of the skin surface of the mothers, while meconium microbiome, akin to maternal vaginal microbiome, is found in the latter group. However, amniotic liquid from mothers who deliver prematurely contains microorganisms independently, whether the membrane is ruptured or intact. This colonization could result from an ascension and translocation of the vaginal microbiota. However, the placenta microbiota in normal healthy term pregnancies is more similar to the oral microbiota than to the vaginal, fecal, skin, or nasal microbiota, suggesting that oral bacteria can penetrate into the uterus environment via the bloodstream (Rodríguez et al., 2015).

### 13.4 CORE MICROBIOME

Subsequently, bacterial community diversity increases and the microbiota is dominated mainly by the phyla *Actinobacteria* and *Firmicutes*. This change in Phase 2 can be attributed to the diet that exerts a profound impact on the composition of gut microbiota, since milk is exclusively consumed by newborns. In breast milk, human milk oligosaccharides (HMO) have prebiotic effects and stimulate the proliferation of *Bifidobacterium* and *Lactobacillus*. During Phase 3, a significant change takes place following weaning and introduction of solid foods; gut microbiota passes from an immature state to that of an adult microbiota characterized by enhanced colonization of butyrate-producing bacteria such as certain *Clostridium* species.

The fecal microbiota, which represents the luminal microbiota well but differs from the mucosal microbiota of the large intestine, contains 1000 species-level phytypes mainly belonging to four major phyla: *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, and *Actinobacteria*. The remaining phytypes belonging to *Verrucomicrobia*, *Fusobacteria*, and *Cyanobacteria* are minor components (Rajilić-Stojanović and de Vos, 2014). Despite the large complexity and high interindividual variability, the composition of a “core” microbiota has been identified based on bacterial phytypes found in fecal samples. The core microbiota contains 66 molecular species, conserved over 50% of a population including *Faecalibacterium*, *Ruminococcus*, *Eubacterium*, *Dorea*, *Bacteroides*, *Alistipes*, and *Bifidobacterium* (Tap et al., 2009; Mondot et al., 2013). In the absence of perturbations and modifying factors such as diet, hormonal cycles, travel, therapies, and illness, the individual dominant microbial composition is relatively stable during adulthood. Fecal microbiota of the elderly is distinct from younger adults; the proportion of *Bacteroidetes* is higher in older people, while the *Firmicutes* phylum is dominant in youths. The *Bacteroidetes*-dominant character is confirmed by a higher relative abundance of the genera *Bacteroides*, *Alistipes*, and *Parabacteroides*. Interindividual variation is also
greater, which is expressed by significant changes in the relative abundance of *Firmicutes* and *Bacteroidetes* (Gerritsen et al., 2011). Alterations of the fecal microbiota of the elderly may be caused by physiological factors such as chronic low-grade inflammation (inflammaging), an increased susceptibility to infections and inflammatory diseases (immunosenescence), as well as nutrition modifications and an increase in medication intake (Guinane and Cotter, 2013). Claesson et al. (2012) have highlighted the relationship between diet and health status with the fecal microbiota of the elderly. The microbiota of older people residing in a long-term care environment is less diverse, with a higher proportion of *Bacteroidetes* than those living in the community.

The enterotype concept has been proposed to stratify the human population in different genetic groups present in the large intestine. Three enterotypes were identified: *Bacteroides* (enterotype 1), *Prevotella* (enterotype 2), and *Ruminococcus* (enterotype 3) (Arumugam et al., 2011; Wu et al., 2011). The term “faecotypes” would be more appropriate than enterotype because the composition and abundance of microbial populations varies considerably throughout the GI tract (Siezen and Kleerebezem, 2011). Moreover, the boundaries between enterotypes are more vague than previously thought. The concept is based on the presence of the most prevalent species, and their specific functions are still unknown. Moreover, this concept does not consider the less abundant species of the fecal microbiota that have fundamental functions in a particular site. In addition, there is a large interindividual variation in the composition of the microbiota, and, at the level of genes coding for specific metabolic functions, many bacterial species share common functions. Therefore there is a high level of redundancy and similarity between persons at a functional level. Hence, if the notion of a “core microbiota” is questionable, that of a “core microbiome” is convincing. It would be wiser to expand the concept of enterotypes to that of “enterogradients,” which takes into account the continuum of microbial community structures to better describe the relative composition of human microbiomes. Thus the “metabolotype” (metabolic phenotype) concept would have a greater meaning. Indeed, enterotypes 1 and 2 (*Bacteroides* and *Prevotella*) belong to the same order *Bacteroidales*, and are grouped together based on their functions in the same metabolotype. The Enterobacteriaceae group forms a second metabolotype, while the third metabolotype includes phyla *Firmicutes*, *Ruminococcus* and *Actinobacteria* (Holmes et al., 2012).

Certain dominant bacterial groups are considered beneficial by promoting gut health. Here we will describe which ones have a strong impact on the health of the host and which possess potential probiotic properties.

### 13.4.1 *BIFIDOBACTERIUM*

Among bacterial groups present in the large intestine, and known to play a role in the wellbeing and health of the host, bifidobacteria occupy a prominent place. Bifidobacteria are Gram-positive, anaerobic, saccharolytic, and pleomorphic rods, clustered within a distinct branch of the Actinobacteria. *Bifidobacterium* spp. is dominant in the fecal microbiota of breastfed infants. The difference between the observed abundance of bifidobacteria in breastfed infants and formula-fed infants is explained by the presence of prebiotics (HMO) and bifidobacteria in the breast milk, suggesting their vertical transfer from the mother’s milk to the child (O’Callaghan and van Sinderen, 2016). During Phase 3 of microbial colonization, bifidobacterial populations can be surpassed by those of other bacterial groups. Their populations are still dominant in adults and decrease among older people. *Bifidobacterium longum* ssp. *longum* is considered one of the 10 most prevalent species in the adult gut. The analysis of the genomes of 47 *Bifidobacterium* species reveals a strong selective pressure to the acquisition and
retention of accessory genes necessary for the degradation and internalization of a wide range of carbohydrates. Therefore bifidobacteria could be competitive in a particular ecological niche, such as the human intestine gut. Bifidobacteria are metabolically active in the lower part of the colon using trophic interactions, which may vary from commensal to mutualistic. They have also access to a wide variety of polysaccharides and oligosaccharides of plant origin that are not digested by the host, as well as host glycans such as mucin and HMO. Their functional contribution to the human gut microbiome through crossfeeding activities can be important in terms of expanding the global glycobiome of the large intestine and affecting the overall physiology of the host intestine (Milani et al., 2015).

13.4.2 FAECALIBACTERIUM PRAUSNITZII

Faecalibacterium prausnitzii is a commensal Gram-positive bacterium, strict anaerobe, isolated from human microbiota and a leading representative of the phylum Firmicutes, class Clostridium, family Ruminococcaceae. *F. prausnitzii* is an acetate-consuming and butyrate-producing microorganism known to possess antiinflammatory effects by secreting metabolites and intestinal homeostasis protection properties. A depletion of *F. prausnitzii* has often been reported in Crohn’s disease (Sokol et al., 2008, 2009).

*F. prausnitzii* is known as a “fecomucus” bacterium. Indeed, faecalibacteria may total 5%–20% of the microbiota of fecal mass, but they are also detected in mucus (Miquel et al., 2014). *F. prausnitzii* populations are distributed throughout the GI tract, particularly in the proximal part of the colon. They are also found in niches with relatively high oxygen tensions as adjacent mucus to epithelial cells. However, *F. prausnitzii* is a bacterium that is extremely sensitive to oxygen, and loses its viability after 2 min of exposure to ambient air. This bacterium would, however, be able to grow under microaerophilic conditions in the presence of flavin and cysteine or glutathione (Khan et al., 2012).

*F. prausnitzii* grows poorly on nondigestible carbohydrates and needs acetate for optimal proliferation and butyrate formation. This suggests the existence of symbiotic cooperation or crossfeeding between *F. prausnitzii* and acetate-producing bacteria as *Bifidobacterium* spp. Bifidobacteria can produce acetate by degradation of fibers and prebiotics that become available to butyrate-producing bacteria such as *F. prausnitzii*. These bacteria are thus able to grow and produce greater amounts of butyrate, which is absorbed by colonocytes as an energy source (Rios-Covian et al., 2015).

13.4.3 AKKERMANSIA MUCINIPHILA

Akkermansia muciniphila is an anaerobe Gram-negative bacterium belonging to the phylum Verrucomicrobia. This mucin-degrading bacterium that resides in the mucus layer represents 1%–4% of the fecal microbiota of healthy people. *A. muciniphila* colonizes the intestinal tract in early life and reaches a level close to what is observed in adults during the first year. Unlike most other members of the fecal microbiota, it is considered as an aerotolerant anaerobic bacterium rather than a strict anaerobe. Members of the genus Akkermansia have been suggested as biomarkers for a healthy gut, because *A. muciniphila* has been linked to intestinal health and improvement of the metabolic condition in obese and type 2 diabetes subjects (Everard et al., 2013). It has been postulated that *A. muciniphila* plays a key role in the pathophysiology of obesity, type 2 diabetes, and metabolic inflammation because the presence of viable *A. muciniphila* within the mucus layer is a crucial mechanism in the control of the host mucus turnover. Furthermore, its ability to increase the mucus layer thickness, which restores
A. muciniphila is able to use the intestinal mucins as a sole carbon and nitrogen source and produce acetate and propionate in the mucus layer, closely related to epithelial cells, making these SCFA easily available for the host. These substrates can also serve as energy sources for other bacteria and contribute to the proliferation of other beneficial species by crossfeeding mechanisms. The administration of prebiotics such as oligofructose and polyphenolic compounds or probiotics such as *Bifidobacterium animalis* ssp. *lactis* LMG P-28149 results in an increased abundance of *A. muciniphila* (Alard et al., 2016; Anhê et al., 2015a,b, 2016; Everard et al., 2013). This can be explained by the production of acetate and lactate by bifidobacteria, which, by crossfeeding, stimulate the growth of *A. muciniphila*.

### 13.5 The Probiotic Concept

Probiotics are available on the market in the form of dietary supplements and functional foods. These microorganisms must remain viable and metabolically active during different steps (preparation, production, and storage) of their incorporation in food products, as well as the time of ingestion to reach the intestine. Beneficial bacteria such as *Lactobacillus* and *Bifidobacterium* can meet such criteria. Lactobacilli are an important component of many traditional foods. These bacteria are also true residents of the gut microbiota, as well as bifidobacteria, and are used as a proven, safe method of contributing beneficial physiological effects on human and animal health. Probiotic strains are mainly chosen for technological reasons because they survive the various stresses encountered during their preparation. Indeed, many promising strains cannot be produced industrially because of low yields in the growth media or poor survival rate when frozen or freeze dried, and cannot be commercialized (Champagne et al., 2005).

Probiotic bacteria are traditionally found in products such as fermented milk, kefir, sauerkraut, or kimchi; yogurt being the most popular among consumers. According to the *Codex Alimentarius*, yogurt is a category of fermented milk product, which contains the starter microorganisms *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*. These starter microorganisms “shall be viable, active and abundant in the product to the date of minimum durability” (Codex standard for fermented milk types, CODEX STAN 243-2003). With respect to this, the yogurt product should contain active bacterial cultures in amounts \( \geq 10^8 \) organisms/g at the time of manufacture and remain active at the end of the stated shelf-life.

The definition of probiotics according to the *Guidelines for the Evaluation of Probiotics in Food* published in 2002 by the FAO/WHO working group has been revised by an expert panel of the International Scientific Association for Probiotics and Prebiotics as “live microorganisms which, when administrated in adequate amounts, confer a health benefit on the host” (Hill et al., 2014). In Canada, a list of eligible bacterial species for Acceptable Non-Strain-Specific Claims for Probiotics includes *Bifidobacterium* (*adolescentis*, *animalis*, *bifidum*, *breve*, and *longum*) and *Lactobacillus* (*acidophilus*, *brevis*, *casei*, *fermentum*, *gasseri*, *johnsonii*, *paracasei*, *plantarum*, *delbrueckii*, *rhamnosus*, *reuteri*, and *salivarius*). The best approach is to include on the label the genus, species, and strain designation for each probiotic in the product and the level of viable cells of each probiotic strain at the end of shelf-life (Pineiro and Stanton, 2007; Sanders et al., 2007).
The incorporation of probiotic bacteria in yogurt requires a rigorous selection of strains based on general microbiological criteria. First, the strains must be identified by phenotypic and genetic tests. With the recent progress, whole-genome sequencing becomes the gold standard for characterization. In addition, this method can determine the pathogenic potential by identifying the presence of virulence genes. Genomic data also help to establish a relationship between genomic sequences and metabolic pathways or putative proteins involved in the mechanisms of action of potentially probiotic strains (Huys et al., 2013). The strains added to probiotic yogurt should also be deposited in an independent reference culture collection to ensure that the product taken by the consumer is consistent with the commercialized product.

Probiotic yogurt quality not only depends on the probiotic strain used, but also on its concentration, which should be sufficient to provide a beneficial effect on the fecal microbiota balance. Canada and Italy have legislated that this level must be of $1 \times 10^9$ colony forming units (cfu) per portion, according to Health Canada, and a minimum number of viable cells ($1 \times 10^9$ cfu) per day for the recommended portion according to the Italian Ministry of Health. To ensure that the yogurt contains this number at the time of consumption, the inoculation rate should be based on the survival capacity of probiotic strains during the manufacture process and storage. Indeed, contrary to yogurt starters, lactobacilli and bifidobacteria are not able to grow at a fermentation temperature of 40–45°C. Unless the incubation temperature is lowered, the inoculation rate is determined by the level of bacteria obtained in the lyophilized or frozen preparation. The inoculation level should also take into account the various factors that can affect the survival of probiotic bacteria such as their high sensitivity to oxygen, their low tolerance to acids from their metabolism, and their nutritional requirements. At the time of manufacture, interactions between bacterial species and dissolved oxygen content, especially for bifidobacteria, are to be considered. During storage, the final acidity and pH values of the product, as well as the postacidification and oxygen during refrigerated distribution and storage, all have a major impact on the survival of probiotics (Champagne et al., 2005). In general, bifidobacteria are sensitive to pH values below 4.6, except the aerotolerant strains of *B. animalis* ssp. *lactis*, which are known to have a tolerance to acid (Jungersen et al., 2014).

In the selection of probiotic strains, additional criteria (such as their ability to resist gastric acids, bile salts, and pancreatic secretions) should be considered to ensure that the strains are able to reach the colon. Gastric acids are the primary defense mechanism against microorganisms, while bile salts and pancreatic juices can also exert antimicrobial activity. The influence of other digestive secretions, such as mucus and enteric secretions, including defensins, is little known. Probiotic concentrations, which are able to reach the intestine alive, are influenced by survival and the amount ingested. Some probiotics are destroyed during their passage through the stomach (pH 1.5–2.0 during fasting and pH 4.0–5.0 postprandial), while others may pass through the small intestine and colon. Their survival rates are determined by in vitro tests. The strain sensitivity to the pH and bile acids is often determined under static conditions. Although these in vitro models are simple and inexpensive, their limitations include dividing the process of digestion into several independent steps and not taking into account the influence of diet and gastric secretions on the gastric transit of microorganisms during digestion. Dynamic models with several compartments using a computer program are closer to the physiological conditions of digestion. However, these dynamic models do not allow for studying adhesion to the intestinal mucosa. Finally, in vivo studies can also be performed in animal models allowing for the evaluation of probiotic survival in real environmental conditions of the GI tract, although disadvantages include their elevated costs and ethical considerations.
Bile acid deconjugation carried out by the bile salt hydrolase (BSH, EC 3.5.1.24) is an activity that may be desirable for probiotic strains, as it could maximize their chances of survival in the GI tract. BSH activity also has beneficial effects on the host, such as the reduction of cholesterol levels. The BSH catalyzes the hydrolysis of bile salts conjugated with glycine or taurine in amino acid residues and free bile salts. However, in excess, this process can be harmful to the host. Many regulatory agencies require an evaluation of BSH activity to ensure that deconjugation in the small intestine will not be increased, and that no change will occur in the large intestine. BSH activity can be determined by a simple verification of the precipitation obtained on Petri dishes following incubation with bile salts. On the other hand, for comparison of BSH activity of a strain with that of another, more specific methods such as chromatographic techniques are preferred (Begley et al., 2006).

One possible beneficial consequence of BSH activity in a probiotic bacterium is the accumulation of cholic acid inside the bacterial cell, released under the action of BSH that also contributes to decrease the secondary bile acid production (Kurdi et al., 2003). Secondary bile salts are cytotoxic and carcinogenic molecules. The enzyme responsible for this undesirable reaction is the 7 alpha-dehydroxylase, which can be found in intestinal bacteria, but not in lactic acid bacteria and bifidobacteria (Tsai et al., 2014).

Resistance to antibiotics of probiotic strains should also be rigorously evaluated to verify the presence of acquired and even potentially transmissible antibiotic resistance traits, and to minimize the risk of horizontal transfer. Probiotic bacteria should therefore be tested for their susceptibility to many antibiotics and only those that are susceptible to more than two major antibiotics should be retained (Gueimonde et al., 2013). In Italy, the safety assessment of each strain is not considered necessary when the strain belongs to a species extensively characterized in terms of safe use, as defined by the European Food Safety Authority (EFSA) document for the Qualified Presumption of Safety status (EFSA, 2012).

### 13.6 TRANSIENT MICROBIOME

A stable microbiota contributes to the intestinal homeostasis by helping to maintain the intestinal barrier integrity functions, immune balance, and inflammation control. In contrast, disturbances of gut microbiota induced by a high-fat, high-sugar (western-type) diet, antibiotic administration, or accidental ingestion of pathogenic microorganisms, lead to increased intestinal permeability, impaired immune function, and chronic low-grade inflammation. A nutritional approach based on long-term consumption of fiber, prebiotics, and probiotic yogurts is possibly able to counter dysbiosis. However, some microbial ecology concepts should be considered to determine whether probiotic strains are able to contribute favorably to modulate the fecal microbiota.

The diversity of microbial communities can be defined as the number (richness or “which species are present”) and abundance distribution (evenness or “how many species are present”) of distinct types of organisms in a specific ecosystem. The diversity of the intestinal microbiota was found to be associated with stability because species-rich communities are less susceptible to invasion and more resistant to change caused by an ecological stress, and with the resilience by the ability of these communities to return to an equilibrium state following stress-related perturbations. These microbial communities use more effectively limiting resources because different species may specialize to metabolize the specific substrates in a given niche. Ecological resilience is lowered after the loss of a species in a community
as a result of stress, such as antibiotic administration that perturbs the microbiota or a diet rich in fat and sugar, which favors the proliferation of species at the expense of others because of the greater abundance of nutrients. On the other hand, microorganisms can promote resilience because they are able to induce beneficial physiological conditions for their growth compared to growth conditions for their potential competitors (Gibson et al., 2014; Greenhalgh et al., 2016; Lozupone et al., 2012; Shade et al., 2012).

The number of bacteria and size of the microbial communities that reside in different parts of the intestine are stable over time because their growth rates in the digesta compensate for the constant loss in feces. The dominant populations represent densities of $10^9$–$10^{12}$ cfu/g of feces while those of subdominant populations represent densities of $10^6$–$10^8$ cfu/g. These residents known as *autochthonous* are well adapted to specific environments of a given intestinal niche and tolerated by the immune system, but are exposed to orally ingested (harmful or potentially beneficial) microorganisms. These are provided mainly by food and can colonize saliva, subsequently the intestine, and disappear within a few hours in fecal matter. One of the main functions of indigenous bacteria is to act as a host’s defense against colonization by ingested microorganisms (known as “colonization resistance” or “barrier effect”). The main source of these exogenous organisms known as *allochthonous* are fermented foods such as yogurt and cheese that may contain significant amounts of live bacteria up to $10^9$ cfu/g. The proportion of daily-ingested viable bacteria can reach $10^{10}$–$10^{11}$ per day, depending on the food habits and geographical areas, and represent 0.1%–1% of the fecal commensal microbiota. Despite the resistance of autochthonous bacterial communities to colonization by allochthonous populations, many bacteria such as probiotic strains are able to pass safely through the stomach, reach the intestine, and affect the composition and activity of the resident gut communities. Derrien and van Hylckama Vlieg (2015) proposed the term “transient microbiome,” whose composition is dynamic and individualized depending on the influence of diet, exposition to ingested probiotic bacteria, environmental conditions of the intestine, and other factors associated with the host. In summary, the open microbial ecosystem of the GI tract is composed of commensal residents and is constantly exposed to exogenous microbes from the diet that will “transiently” be part of this ecosystem (Derrien and van Hylckama Vlieg, 2015; Zhang et al., 2016).

Probiotic bacteria can act on fecal microbiota by various mechanisms. Bifidobacteria, for example, are able to produce lactic acid and acetic acid from the degradation of polysaccharides present in food such as arabinoxylans or inulin. By crossfeeding, acetate or lactate is used by butyrate-producing bacteria that proliferate and release butyrate in the intestinal environment (O’Callaghan and van Sinderen, 2016; Rivière et al., 2013, 2015). Probiotic strains may have a prebiotic effect by selectively stimulating other endogenous lactobacilli and bifidobacteria. Probiotic bacteria may also induce physicochemical changes by locally modifying the intestinal environment. A decrease in pH may promote the growth of beneficial bacteria or interfere with undesirable bacteria. The production of antimicrobial compounds such as bacteriocins can control pathogenic organisms, while vitamin synthesis may promote the growth of commensal bacteria. Probiotics can also interact with the host’s epithelial cells, change the composition of mucus secreted by colonocytes, and strengthen the tight junctions between epithelial cells. Finally, probiotics bacteria can modulate, directly or indirectly, the immune system toward proinflammatory or antiinflammatory action, and induce the production of antimicrobial peptides (including defensins and cathelicidins) by the host (Gerritsen et al., 2011).
13.7 **HEALTH CLAIMS**

Although the characteristics of compositions and functions of the bacterial community in the intestines of healthy people remain to be defined, it seems that transient microbes such as orally ingested probiotic bacteria can induce changes in the composition of the intestinal microbiota, directly or indirectly modify the metabolic production of SCFA (butyrate, acetate, and propionate), and modulate the immune system functions as immunomodulation and immunostimulation. However, the contribution of probiotic yogurts on the human intestinal microbiome is still relatively unknown as well as benefits for the host, except for two health claims approved by regulatory authorities.

In 2011, EFSA recognized health claims related to live yogurt cultures (\textit{L. delbrueckii} ssp. \textit{bulgaricus} and \textit{S. thermophilus}), which are able to compensate for the deficit of lactase in adults and improve lactose digestion \cite{Scott2015}.

In 2013, Switzerland’s Federal Office of Public Health authorized the following health claim: “Activia® contributes to digestive comfort by reducing transit time and bloating.” Danone’s Activia yogurt, containing \textit{B. animalis} (lactis) DN-173 010, has been tested in two randomized, double-blind, controlled, and parallel-group investigations. The goal of these studies was to determine if women who were regularly fed fermented milk containing probiotic \textit{B. lactis} CNCM I-2494 (\textit{B. lactis} DN-173 010) with lactic acid bacteria (\textit{L. delbrueckii} subsp. \textit{bulgaricus} CNCM I-1632 and I-1519, \textit{S. thermophilus} CNCM I-1630, and \textit{Lactococcus lactis} ssp. \textit{lactis} CNCM I-1631) experienced a greater sense of GI wellbeing than those who received a nonfermented dairy product (without the probiotic with a low content in lactose and acidified using an enzymatic process) after 4 weeks’ consumption of this probiotic product. The test product contained $1.25 \times 10^{10}$ cfu of \textit{B. lactis} DN-173 010 per portion (125 g) and $1.2 \times 10^{9}$ cfu of \textit{S. thermophilus} and \textit{L. delbrueckii} ssp. \textit{bulgaricus} per portion. The design of the two studies allowed subjects to be recruited within the general population. The subjects had no diagnosed GI disorders on the basis of the presence and frequency of these minor digestive symptoms. Endpoints comprised weekly assessment of GI wellbeing (primary outcome), rate of responders, and digestive symptoms \cite{Guyonnet2009, Marteau2013}.

In the first study, the digestive comfort score of women who were fed Activia yogurt twice a day for 4 weeks was significantly higher than that of the control group \cite{Guyonnet2009}. In the second study, no significant difference was observed in the percentage reporting an improvement in GI wellbeing. However, in the pooled analysis, significant differences were observed with all endpoints (GI wellbeing, rate of responders, and composite score of digestive symptoms) \cite{Marteau2013}. In the first study, the overall symptom improvements were mainly associated with rumbling (borborygmi) and flatulence, but no significant score was observed for bloating and abdominal pain/discomfort. The effect of the probiotic product was not maintained after ceasing its consumption. Combining the results of two clinical trials in a pooled analysis allowed validation of the beneficial effect of the daily consumption of Activia on global assessment of GI comfort, and decreasing in frequency of digestive symptoms in a population of women reporting minor digestive issues, although the benefits could be considered as modest.

Bowel habits include four characteristics: stool frequency, consistency, weight, and transit time. Colonic disturbances are frequent in healthy populations. With aging, a reduced intestinal motility can cause constipation. Activia yogurt, containing \textit{B. lactis} DN-173 010, has been tested for regularity of bowel movement in healthy women and elderly people (Table 13.1). These studies indicated that the probiotic yogurt shortened the transit time in women, but did not influence fecal excretion of secondary
### Table 13.1 Clinical Studies on the Impact of *Bifidobacterium animalis* ssp. *lactis* DN-173-010 on Gut Transit Time of Healthy Women and Elderly

<table>
<thead>
<tr>
<th>Method (Trial Design)</th>
<th>Participants or Subjects</th>
<th>Daily Dose and Frequency</th>
<th>Treatment Characteristics</th>
<th>Duration of Treatment</th>
<th>Measures of Efficacy, Results, and Conclusions</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parallel double-blind study</td>
<td>72 healthy volunteers with a mean age of 30 (range 21–42 years; 36 males and 36 females)</td>
<td>Product A (test group): Milk fermented with <em>Bifidobacterium animalis</em> (DN-173 010 strain) at a concentration of $2.6 \times 10^8$ cfu/g. Product B (control group) was prepared as the product A heat treated to contain no viable <em>Bifidobacterium</em>.</td>
<td>During a period preceding the intervention (D01–D10), usual fermented milk and yogurt consumption were excluded. Products A (treatment) and B (control) were added to the diet during D10–D21 period (3 × 125 g daily). Treatment group (A) received $9.75 \times 10^{10}$ cfu of the probiotic bacteria daily.</td>
<td>11 days</td>
<td>Endpoints or measures of efficacy: The main marker was the total colonic transit time (CTT) measured with radioopaque pellets. X-rays of abdomen were done on D10 and D21 and the radioopaque markers were counted in each segment of the colon. Effects or results: Within the treated group, total CTT was reduced by 20.6% ($P = .013$) and sigmoid transit time (TT) was reduced by 38.9% ($P = .02$) between D10 and D21, whereas right and left CTTs tended to be shorter, but not enough to reach statistically significant differences. Within the control group, there were no significant variations of TTs. The difference between initial and final total CTT was significantly ($P &lt; .05$) greater in the bifidus group (−6.8 h) than in the control group (+0.5 h). Conclusions: A milk fermented with <em>B. animalis</em> DN-173 010 was able to shorten CTT in humans, mainly because of improvement of sigmoid TT.</td>
<td>Bouvier et al. (2001)</td>
</tr>
</tbody>
</table>

*Continued*
Table 13.1 Clinical Studies on the Impact of *Bifidobacterium animalis* ssp. *lactis* DN-173-010 on Gut Transit Time of Healthy Women and Elderly—cont’d

<table>
<thead>
<tr>
<th>Method (Trial Design)</th>
<th>Participants or Subjects</th>
<th>Daily Dose and Frequency</th>
<th>Treatment Characteristics</th>
<th>Duration of Treatment</th>
<th>Measures of Efficacy, Results, and Conclusions</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Randomized double-blind, crossover study</td>
<td>36 healthy women aged 18–45 years without constipation</td>
<td>The test product: Milk fermented with <em>B. animalis</em> (DN-173 010 strain) at a concentration between $5 \times 10^7$ and $10^8$ cfu g. The control (placebo) product was the same fermented milk with yogurt cultures without bifidobacteria.</td>
<td>Four consecutive periods: A 10-day run-in period and two 10-day ingestion periods with an interval of 10 days between them. During the ingestion period, the subjects received three 125 g cups per day of either control product ($3.75 \times 10^{10}$ yogurt cultures) or the tested product containing yogurt cultures plus the bifidus supplement (between 1.87 and $7.5 \times 10^{10}$ <em>B. animalis</em>). Half of the subjects received the control before bifidus (referred as group A) and half received bifidus before the control (group B).</td>
<td>Two 10 days with an interval of 10 days between them</td>
<td>Endpoints or measures of efficacy: Stool analysis was performed in 12 of the volunteers; all stools in the last 3 days of each study period were collected. In the last 3 days of the run-in and each test period, the subjects ingested every day 20 radioopaque pellets. Effects or results: The CTT and the sigmoid TT were significantly shorter after bifidus than after control consumption. In the whole population, CTT significantly decreased from 55.2 to 51.5 h (60.7 h for the control group) and in the subgroup with an initial TT above 40 h, the CTT significantly decreased from 70.4 to 62.4 h (71.9 h for the control) (both $P &lt; .05$). The number of stools per week did not significantly differ between the bifidus, control, and run-in periods (7.0 and 7.0 stools/week). In the 12 subjects analyzed, fecal weight did not differ significantly between the bifidus and control periods and the products had no significant effect on the fecal bacterial mass. Conclusions: <em>B. animalis</em> DN-173 010 shortens the CTT in healthy women, but it did not influence fecal excretion of secondary bile salts and it did not influence fecal weight or bacterial mass.</td>
<td>Marteau et al. (2002)</td>
</tr>
</tbody>
</table>
Randomized, controlled open study

100 suburban free-living elderly (age ranging between 60 and 75 years) with normal clinical and nutritional examinations and regular stool frequency.

The test product: Milk fermented with *B. animalis* DN-173 010 (at least $10^8$ cfu/g).

Two groups of subjects were constituted. One group of 50 subjects with initial stable orofecal TT < 40 h and one group of 50 subjects with an initial TT > 40 h. Those subjects were randomly assigned to consume during 2 weeks either two or three servings per day of 125 g of bifidobacteria fermented milk ($2.5 \times 10^{10}$ or $3.75 \times 10^{10}$ cfu total bifidobacteria).

2 weeks

Endpoints or measures of efficacy: Three consecutive measurements of orofecal TT were done for each subject before and at the end of the product intake period. Colored markers were used to assess orofecal TT.

Effects or results: The consumption of either two or three servings of bifidus milk (BM) during 2 weeks decreased significantly the orofecal TT. In subjects with a TT < 40 h, the TT decreased 1.7 h with 2 BM and 2.9 h with 3 BM; in those with a TT > 40 h, the TT decreased 24.6 h with 2 BM and 28.6 h with 3 BM. Postvalues statistically differed from prevalues ($P < .001$) and 2 MB values were significantly different from 3 BM ($P < .05$).

Conclusions: A 2-week regular consumption of milk fermented with *B. animalis* DN-173 010 and lactic cultures shortened in a dose-dependent manner TT in elderly, especially in those with longer TT.

Randomized

200 healthy volunteers aged between 50 and 75 years, with normal clinical and nutritional examination, and regular stool frequency as well as stable orofecal transit time.

A commercial test product marketed under the names of Bio or Activia (provided by Danone, France), a dairy product fermented by the strain *B. animalis* DN-173 010 plus yogurt cultures (BM). The population of bifidobacteria was equal to or at least $10^8$ cfu/g. The population of yogurt cultures (*Streptococcus thermophilus* and *Lactobacillus bulgaricus*) was equal to at least $10^7$ cfu/g.

100 subjects with a medium orofecal TT (40–50 h) and 100 with a slow TT (> 50 h) were randomized to one of two groups (A or B). Subjects in group A, when not receiving treatment, acted as a control for those in group B and vice versa. Following a 7-day run-in period, subjects in group A received 125 g of BM ($1.25 \times 10^{10}$ cfu of bifidobacteria) daily for 2 weeks, followed by a 6-week follow-up period. Subjects in group B received no active treatment for a control period of 4 weeks following the run-in period. At the end of this period, they received 250 g of BM daily ($2.5 \times 10^{10}$ cfu of bifidobacteria) for 2 weeks, followed by a 6-week follow-up period.

2 weeks

Endpoints or measures of efficacy: Total orofecal TT was measured in each subject every 2 weeks. TTs were determined using colored markers.

Effects or results: Consumption of BM significantly reduced TT in both medium TT and slow TT groups. With one serving of BM (group A), mean TT was reduced by 21% ($P < .05$) compared with initial values and compared with the control group. In group B, TT was significantly reduced, with a 42% reduction to 26.9 h after 14 days in medium TT subjects. Similarly, statistically significant reductions in TT were observed in the slow TT group, with around a 27.2% reduction with one 125 g serving of BM daily (group A), and a 38.1% reduction compared with initial values. Importantly, the positive effect of BM on intestinal TT continued during the follow-up period in both groups, from 2 to 6 weeks.

Conclusions: This study demonstrated that consumption of fermented milk incorporating the probiotic strain *B. animalis* DN-173 010 for 2 weeks dose dependently reduced orofecal TT in elderly subjects, especially in those with longer initial TTs.

Meance et al. (2001)

Meance et al. (2003)
bile salts. It did not influence fecal weight or bacterial mass transit time in the elderly, especially for those with longer transit times (Bouvier et al., 2001; Meance et al., 2001, 2003; Marteau et al., 2002).

In summary, fermented milk and yogurt products are ideal vehicles for the delivery of probiotic bacteria, and are perceived positively by consumers for the following reasons: (1) the positive image of healthy food associated with fermented dairy products; (2) the fact that consumers are aware that conventional yogurt contains active bacteria (yogurt starters); and (3) the incorporation of probiotic strains combines the physiological benefits (lactose maldigestion) of fermentation by yogurt starters and those of probiotic cultures (control transit time and bowel habits).

13.8 PROBIOTIC YOGURT INTAKE

Stability of the microbial community structure can be achieved through increased levels of beneficial bacteria such as *Bifidobacterium* and *Lactobacillus*, preferably at the expense of more harmful bacteria. Therefore before addressing the capacity of probiotic yogurts to modulate the fecal microbiota, it is better to know which doses can increase the level of bifidobacteria or lactobacilli in fecal samples. Determining the amount of bacteria recovered from the feces before and after ingestion of probiotic yogurt is a valid marker to compare the impact of different strains if the dose levels are comparable. Appropriate microbiological methods can assess the increase in specific probiotic strain in fecal microbiota.

We carried out a randomized, placebo-controlled, double-blind, parallel dose–response study to investigate the effect of yogurt supplemented with *B. animalis* ssp. *lactis* (BB-12) and *L. acidophilus* (LA-5) on their recovery from feces (Savard et al., 2011). The primary outcome of this intervention study was to quantify changes from baseline in the fecal number of *L. acidophilus* LA-5 and *B. lactis* BB-12 after daily consumption of yogurt with $10^9$ cfu/100 g of BB-12 and LA-5 or yogurt with $10^{10}$ cfu/100 g of BB-12 and $10^9$ cfu/100 g of LA for 4 weeks. The number of total anaerobic bacteria, bifidobacteria, lactobacilli, *B. lactis*, and LA-5 was estimated by quantitative polymerase chain reaction (qPCR). We observed that the bifidobacterial population was higher for both doses, compared to placebo after 4 weeks of yogurt intake. We did not observe any significant changes for total bacteria and lactobacilli by qPCR in agreement with our results using the plate-counting techniques. Indeed, we did not detect any significant differences for total bacteria, bacteroides, and clostridia between probiotic yogurt and placebo treatments. However, plate counts of lactobacilli were significantly higher, whereas those of enterococci significantly decreased after 28 days of probiotic yogurt intake.

After the run-in period of 1 week, we detected *B. lactis* in 31/55 subjects and LA-5 in 4/55 above the detection limit of $4.5 \log_{10}$ cfu/g. In the placebo treatment, the presence of both probiotics in feces decreased after the intake period, whereas after 4 weeks of probiotic yogurt consumption, *B. lactis* was detected in 100% of the subjects (Table 13.2) at a concentration around $8.5 \log_{10}$ cfu/g regardless of the dose taken. The detection percentage for *L. acidophilus* LA-5 was between 68% for the lower dose of BB-12 ($10^9$ cfu) and 83% for the higher dose ($10^{10}$ cfu). In addition, the LA-5 number was slightly higher ($5.8 \log_{10}$ cfu/g) at the higher dose of BB-12, as compared to the lower dose ($5.8 \log_{10}$ cfu/g).

We concluded that the concentration of $10^9$ cfu per portion of both probiotic strains was sufficient to have an impact on their concentration in feces. Our study validated that BB-12 and LA-5 can survive the “hostile” environment found in the intestinal tract, as evidenced by the detection of the BB-12
<table>
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<tr>
<th>References</th>
<th>Product, Bacteria, and Dose (cfu/day)</th>
<th>Number and (age years old)</th>
<th>Trial Design and Duration (days)</th>
<th>Methods</th>
<th>Effects on Fecal Microbiota</th>
<th>Percentage of Probiotic Detection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Savard et al. (2011)</td>
<td>100 g yogurt daily <em>Lactobacillus acidophilus</em> LA-5, 8 × 10⁸ and <em>Bifidobacterium lactis</em> BB-12, 5 × 10⁹, 5 × 10¹⁰</td>
<td>58 (18–54)</td>
<td>Randomized, double-blind, placebo-controlled (28)</td>
<td>Plate count qPCR</td>
<td>+Bifidobacteria (only qPCR), lactobacilli (only plate count), probiotic</td>
<td>ND</td>
</tr>
<tr>
<td>Filteau et al. (2013)</td>
<td>100 g yogurt daily <em>L. acidophilus</em> LA-5, 8 × 10⁸ and <em>B. lactis</em> BB-12, 5 × 10⁹, 5 × 10¹⁰</td>
<td>58 (18–54)</td>
<td>Randomized, double-blind, placebo-controlled (28)</td>
<td>qPCR T-RFLP</td>
<td>+Bifidobacteria, lactobacilli</td>
<td>ND</td>
</tr>
<tr>
<td>Schiffrin et al. (1995)</td>
<td>120 mL of fermented milk three times daily <em>B. lactis</em> BB-12, 1 × 10¹⁰ or <em>Lactobacillus johnsonii</em> LA-1, 7 × 10¹⁰</td>
<td>28 (23–62)</td>
<td>Feeding 21</td>
<td>Plate count API</td>
<td>+Bifidobacteria, lactobacilli</td>
<td>7% (acido)</td>
</tr>
<tr>
<td>Matsumoto et al. (2000)</td>
<td>100 g yogurt daily <em>B. lactis</em> LKM 512 (BB-12), 5 × 10⁹</td>
<td>11 (50–93)</td>
<td>Feeding 14</td>
<td>Plate count API</td>
<td>+Bifidobacteria</td>
<td>56% (lactis)</td>
</tr>
<tr>
<td>Alander et al. (2001)</td>
<td>125 mL low fat yogurt twice a day <em>B. lactis</em> BB-12, 3 × 10¹⁰</td>
<td>30 (22–47)</td>
<td>Feeding 14</td>
<td>Plate count API</td>
<td>+Bifidobacteria</td>
<td>0%</td>
</tr>
<tr>
<td>Mättö et al. (2006)</td>
<td>200 g ABC yogurt daily <em>B. lactis</em> BB-12, 1 × 10¹⁰, <em>Lactobacillus paracasei</em> F19, 2 × 10¹⁰ and <em>L. acidophilus</em> NCFB 1748, 2 × 10¹⁰</td>
<td>14 (36–74)</td>
<td>Routine diagnostic colonoscopy 10</td>
<td>Plate count RAPD</td>
<td>+Bifidobacteria</td>
<td>0%</td>
</tr>
<tr>
<td>Palaria et al. (2012)</td>
<td>94 g synbiotic drinkable yogurt daily <em>B. lactis</em> BB-12, 10⁹–10¹⁰</td>
<td>46 (mean 31)</td>
<td>Double-blind, crossover, placebo-controlled, randomized-feeding (2×21)</td>
<td>qPCR EMA-qPCR</td>
<td>+BB-12</td>
<td>0%</td>
</tr>
<tr>
<td>Matijašić et al. (2016)</td>
<td>100 g synbiotic yogurt daily <em>L. acidophilus</em> LA-5, 1.8 × 10⁹ and <em>Bifidobacterium animalis</em> BB-12 2.4 × 10⁹</td>
<td>30 (18–65)</td>
<td>Double blind, randomized, placebo-controlled multicenter (2×21)</td>
<td>qPCR RAPD seq.</td>
<td>+L. acidophilus LA-5 and <em>B. lactis</em> BB-12 No shift in microbial community structure</td>
<td>25% (acido)</td>
</tr>
<tr>
<td>Veiga et al. (2014)</td>
<td>2×125 g fermented milk product (FMP) daily <em>B. lactis</em> CNCM I-2494 (DN-173 010) 2.5 × 10¹⁰</td>
<td>28 (20–69)</td>
<td>Randomized, double-blind, controlled, parallel-group (28)</td>
<td>Whole-seq.</td>
<td>+Genes of FMP species</td>
<td>ND</td>
</tr>
<tr>
<td>McNulty et al. (2011)</td>
<td>FMP daily <em>B. lactis</em> CNCM I-2494 (DN-173 010) 2.5 × 10¹⁰</td>
<td>7 (21–32)</td>
<td>Seven monozygote female twin pairs (49)</td>
<td>Whole-seq. qPCR seq. RNA seq.</td>
<td>+Butyrate-producers −<em>Bilophila wadsworthia</em></td>
<td>ND</td>
</tr>
</tbody>
</table>

acido, acidophilus: B., Bifidobacterium; bifido, bifidobacteria; EMA-qPCR, ethidium monoazide bromide followed by qPCR; L., Lactobacillus; lactis, animalis ssp. lactis; ND, no data; para, paracasei; qPCR, quantitative PCR; RAPD, random amplification of polymorphic DNA; Seq., 16S rRNA high-throughput sequencing; T-RFLP, terminal restriction fragment length polymorphism; TTGE, temporal temperature gradient gel electrophoresis; Whole-seq., whole genome sequencing.
strain, which was found in 100% of the subjects, and the greater presence of LA-5 in feces after probiotic yogurt intake. *B. lactis* is recognized to survive gastric transit because of acid tolerance and can survive passage through the GI tract into the feces. Marteau et al. (1997) tested the survival of four strains of *B. lactis, L. acidophilus, L. delbrueckii ssp. bulgaricus*, and *S. thermophilus* using an in vitro dynamic model. They showed that *B. lactis* and *L. acidophilus* are more resistant to the passage of the stomach, the passage of the small intestine and the presence of bile salts than *L. delbrueckii ssp. bulgaricus* and *S. thermophilus*. The results obtained with the two probiotic strains are consistent with those in humans using an intubation technique (Marteau et al., 1992). Survival of *B. lactis* present in fermented milk was evaluated using in vivo ileal perfusion (Pochart et al., 1992). The average rate of *Bifidobacterium* sp. recovered in the terminal ileum after 8h was 23.5 ± 10.4% of the amount ingested, and was in agreement with the survival rate estimated at 37.5% in the ileum (Marteau et al., 1992) and a fecal recuperation of *Bifidobacterium* sp. of 30% following administration in fermented milk (Bouhnik et al., 1992).

In most clinical studies, the authors observed an increase of *B. lactis* detection following probiotic yogurt intake (Table 13.2). In the early works on probiotic yogurt intake, Schiffrin et al. (1995) found that the bifidobacteria present in the fecal samples of the subjects during the feeding period corresponded to the BB-12 strain added to the yogurt, but was not detected in the feces prior to feeding. Matsumoto et al. (2000) also observed a significant increase in bifidobacteria numbers following *B. lactis* LKM 512 (BB-12) yogurt intake in the fecal microbiota of 11 subjects, but no significant changes in bifidobacteria during the placebo yogurt intake. The *B. lactis* strain was detected at a high rate during the 2-week consumption period and reached a level of 10^9 cfu/g of wet feces. According to Matsumoto et al. (2000), this strain became the predominant *Bifidobacterium* species by occupying 10% of the total fecal microbiota because *B. lactis* was not damaged by the gastric and bile acids, and reached the colon alive because of its oxygen and acid tolerance. Alander et al. (2001) detected *B. lactis* BB-12 by random amplification of polymorphic DNA (RAPD) at a high rate (100%) in 10 subjects following a 2-week feeding period of 125 mL of yogurt twice a day, but not after feeding. The number of BB-12 in their fecal samples was 6.3 log_{10} cfu/g before the feeding period, 8.6 log_{10} cfu/g during the feeding period, and returned to the starting level 2 weeks after feeding. These results were confirmed by those of Mättö et al. (2006) who detected the same RAPD type of *B. lactis* BB-12 in the fecal samples of 79% of the 14 study subjects at numbers between 5 and 9 log_{10} cfu/g. More interestingly, these authors detected isolates of *B. lactis* BB-12 in biopsy samples of two out of four subjects immediately after probiotic ingestion and in one subject 18–19 days after the end of probiotic ingestion. In this subject, the *B. lactis* BB-12 RAPD type was found in all four fecal samples taken during or after ingestion of the probiotic yogurt, and the numbers of the strain were of 8 log_{10} cfu/g in the sample taken just before the colonoscopy. Finally, our results were confirmed by Matijašić et al. (2016) who found a significant increase in *L. acidophilus* La-5-like bacteria and *B. lactis* by qPCR analysis during the consumption period of symbiotic yogurt containing both probiotic strains but not in the placebo group.

Many studies indicate that *B. lactis* can be isolated from fecal or biopsy samples only for a few days after probiotic yogurt intake is discontinued. Palaria et al. (2012) noted that, although the strain BB-12 was detected in the feces of 70% of subjects during the feeding period with the probiotic yogurt, only 2 out of 22 subjects (9%) contained *B. lactis* after 1 week of washout, and none after 2 weeks. These results are in agreement with those of Mättö et al. (2006) who observed that *B. lactis* BB-12 was in 40% of samples taken 3–4 days after probiotic feeding, but was washed out later (8–9 or 18–19 days).
The only exception was the detection of *B. lactis* BB-12 in a fecal sample of one subject 18–19 days after the feeding period. The detection of this strain in the biopsy samples of this subject suggests prolonged colonization of *B. lactis* BB-12 in the intestines. For *L. acidophilus* La-5, Matijašić et al. (2016) reported similar results. After 1 week of follow-up of symbiotic yogurt consumption, the number of *L. acidophilus* La-5 and *B. lactis* decreased, reaching a level close to the initial level. The persistence of allochthonous bacteria in the intestines is limited because they are in competition with the autochthonous bacteria for nutrient substrates and membership sites. The production of antibacterial compounds by members of the fecal microbiota and others by the immune system probably influences the persistence of probiotics.

Although it has been shown that intake of probiotic yogurt increases bifidobacteria and lactobacilli in exogenous fecal microbiota in the first part of our work (Savard et al., 2011), we had not yet determined whether the proportion of autochthonous bifidobacteria and lactobacilli was affected. More importantly, it is unclear whether the probiotic ingestion modifies the robustness of the bacterial community profile of fecal samples. We assessed the impact of probiotic intake on the structure and diversity of the microbiota as well as the effect on the abundance of bacterial groups on fecal DNA samples obtained during our clinical trial of a commercial yogurt (Savard et al., 2011) using two culture-independent molecular methods: terminal restriction fragment length polymorphism (T-RFLP) and qPCR (Filteau et al., 2013). Based on our qPCR results, we observed in this second part of our study a significant increase in *B. lactis* and *L. acidophilus* LA-5 in DNA fecal samples during treatment. However, neither qPCR quantification nor T-RFLP profiling showed any other modification for the specific bacterial and endogenous bifidobacterial indicators. Our results supported the hypothesis that probiotic consumption does not disturb the robustness of the intestinal microbial ecosystem of healthy adults.

Palaria et al. (2012) also found that *B. lactis* increased after BB-12 yogurt ingestion, whereas the increase of total bifidobacteria was significant only for the subgroup of subjects with bifidobacterial populations lower than $10^8$ cfu/g. Based on qPCR quantification, Matijašić et al. (2016) confirmed that probiotic yogurt intake did not significantly influence the total amount of endogenous lactobacilli or bifidobacteria in the feces, whereas the amount and proportion of La-5 and *B. lactis* increased in subjects in the symbiotic group. These authors estimated that the relative abundance of the ingested probiotic strains in the total fecal microbiota was $<1\%$ for *B. lactis* (median ratio) and $<0.1\%$ for *L. acidophilus* LA-5. Based on their results by metataxonomics obtained using next-generation sequencing, they concluded that the fecal microbiota global profile was not disturbed by the symbiotic yogurt intake. Also no significant shifts in the average abundance of total lactobacilli or bifidobacteria in the feces were noted, while the difference in abundance of *L. acidophilus* LA-5 and *B. lactis* by qPCR could not be confirmed by metataxonomics.

By using a whole-genome sequencing analysis coupled with a gene-centric approach, Veiga et al. (2014) showed that the probiotic yogurt intake containing *B. animalis* ssp. *lactis* CNCM I-2497 increased the level of potential butyrate-producing bacteria and butyrate concentrations in vitro, and decreased the level of an opportunistic pathogen *Bilophila wadsworthia*. McNulty et al. (2011) evaluated the impact of the same fermented milk product on the gut microbiome and observed no changes in the abundance of *B. animalis*, in either humans or mice. Probiotic yogurt intake did not change the overall intestinal bacterial composition, but instead induced definitive changes in the microbiota expression patterns such as increases in carbohydrate and nucleotide metabolism and decreases in amino acid and lipid metabolism.
13.9 CONCLUSION

Probiotic yogurt intake is a major source of allochthonous bacteria. These, in particular *B. lactis*, are able to survive passage through the stomach and reach the intestine at levels of $8 \log_{10} \text{cfu/g}$ to temporarily integrate the fecal microbiota and constitute part of the transient microbiome. There is increasing evidence that probiotic bacteria are biologically active in the colon and could contribute to the intestinal microbial community function by metabolic activities and crossfeeding mechanisms. The persistence of probiotic bacteria is influenced by large interindividual variations in the robustness of the overall ecological gut microbial community. Changes in the metatranscriptome of fecal microbiota are transient, limited by the time of probiotic consumption. Therefore regular consumption of probiotic yogurt is required to obtain digestive health benefits. Future research on the microbiome may contribute toward the emergence of new probiotic strategies to promote wellbeing and health.

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REFERENCES


REFERENCES


SYNBIOTIC YOGURTS AND THE ELDERLY

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14.1 INTRODUCTION

Aging represents a challenge for all sections of society even though the exact definition of the chronological age of “elderly” is controversial. The loss of roles in society because of the decline of physical conditions can determine oldness (Gorman et al., 1999). According to WHO (2016), most developed countries accept the age of 65 years as a definition of elderly, but this does not apply to Africa, for example. Often, “old age” is associated with the age that people receive pension benefits. At the moment, the United Nations does not have a standard numerical criterion but in general the cutoff is 60+ years.

The aging phenomenon occurs quickly around the world with the decrease of fertility and life expectancy. In 2015, it was determined that 901 million people were over 60 years of age, which is 12% of the global population, with an expected growth of 3.26% per year. Europe has the biggest population of people who are 60 or more years old (24%), but by 2050 the expectation is that most countries will have a quarter of their population over 60 years of age (United Nations, 2015).

Significant gains in global life expectancy have been achieved in recent years. The world average, around 70 years in 2014, is 6 years more than in 1990. Africa is the continent where the greatest increase was observed: 6 years in the 2000s compared to a rise of 2 years in the 1990s. In developed countries there has been an impressive increase, with an average of 80 years of life expectancy (Jin et al., 2015; United Nations, 2015).

Demographic and epidemiological changes, along with rapid urbanization, globalization, and changes in risk factors, living standards, and socioeconomic development, have increased the emphasis on chronic diseases identified as centrally associated with the health problems of older people (Suzman et al., 2015; WHO, 2015) and the consideration of degenerative processes as the underlying cause for noncommunicable diseases, such as cancer, cardiovascular disease, type 2 diabetes, Alzheimer disease, arthritis, and others. These factors are also major causes of disability, apart from the addition of and longer exposure to risk factors for noncommunicable diseases, such as tobacco use, an unhealthy and unbalanced diet, excessive use of alcohol, and lack of physical inactivity (Jin et al., 2015), in addition to several comorbid conditions and functional limitations that older persons are likely to have (Tanjani et al., 2015).
The definition of health, given by the WHO in 1948, states that “a state of complete physical, mental and social well-being and not merely the absence of disease or infirmity” is considered to be limited, underestimating the chronic state of some diseases (Jaspers et al., 2015). The assessment of health is a complex issue with a multidisciplinary meaning, including physical, social, mental, and physiological aspects (Tanjani et al., 2015). Aging at the biological level is associated with accumulated cellular and molecular damage, which over time will reduce the overall effectiveness of the organism and increase the risk of developing several diseases. Besides inherent features of physical and mental capacity, the place they live and their interactions also affect the functional competence of individuals. The First World Report on Aging and Health also defines healthy aging “as the process of developing and maintaining the functional ability that enables wellbeing in older age” (WHO, 2015).

Promotion of healthy aging and systems that include the elderly in health, technology, and safety issues means enduring investments for the future, so that this population can gain extra years of life with good health in a friendly environment and not produce a negative effect on society, because advanced age does not mean poor health. Moreover, while the population lives longer, the question remains whether future generations will be healthier than those that came before (Jin et al., 2015; Suzman et al., 2015; WHO, 2015).

An elderly population tends to have more chronic diseases, therefore some additional care is needed, for example, food and nutrition and physical activities (Shrivastava et al., 2013). In this context, for the promotion of healthy aging a safe and efficient strategy is the dietary inclusion of probiotic, prebiotic, or synbiotic foods. Therefore this chapter will discuss the potential of probiotics, prebiotics, and synbiotics in promoting healthy aging and helping to improve the quality of life of the elderly population.

14.2 PROBIOTICS ON THE ELDERLY POPULATION

Probiotics are “live microorganisms, which when administered in adequate amounts, confer a health benefit on the host” (Hill et al., 2014). They are considered functional foods, once they stimulate the immune system and promote resistance to gastrointestinal colonization by pathogens through the production of acetic and lactic acids, bacteriocins, and other antimicrobial compounds (Sanders, 2003).

However, for an organism to be considered a probiotic, the bacterium should be available in high concentrations at the time of intake of the product and remain viable after passage through the gastrointestinal tract (Jensen et al., 2012; Klu and Chen, 2015). The main bacteria used as probiotic supplements are those belonging to the genera *Lactobacillus* spp. and *Bifidobacterium* spp., and to a lesser extent *Enterococcus* spp. (Charteris et al., 1998; Bielecka et al., 2002).

Evidence suggests a positive effect of administration of probiotic supplements on elderly immune response, since aging is associated with immunosenescence and changes in the intestinal microbiota (Nova et al., 2007; Patel et al., 2014). Studies have shown that the administration of probiotic supplements can modify the intestinal microbiota in favor of beneficial composition and hence improve the immune function in these individuals (Tiihonen et al., 2010).

Table 14.1 presents some clinical studies showing the effects of probiotics on elderly individuals.

After 3 weeks of supplementation with *Bifidobacterium lactis* HN019 (10⁹–10¹⁰ cfu/day), Chiang et al. (2000) observed in the elderly an increase in phagocytic capacity of monocytes and natural killer cell activity. Similar results were reported by Sheih et al. (2001) from supplementation of elderly and middle-aged subjects with *Lactobacillus rhamnosus* HN001 strains for 3 weeks (Sheih et al., 2001).
Ouwehand et al. (2008) administered strains of *Bifidobacterium longum* 2C and 46 and *B. lactis* Bb-12 to the elderly and observed a reduction of the inflammatory state. Turchet et al. (2002), when evaluating the effect of a fermented milk probiotic by using strains of *Lactobacillus casei* DN-114001 for 3 weeks on the incidence of winter infections in elderly individuals, observed that there was a reduction in the duration of infections in the group treated with the probiotic.

Further, several studies reported that supplementation with probiotics in elderly individuals has positive effects on intestinal functions, such as reducing the time of the oral–fecal transit and increased evacuation frequency (Meance et al., 2001; Ouwehand et al., 2002; Pitkälä et al., 2007).

The use of probiotics can also be related to the prevention of diarrhea associated with the use of antibiotics in the elderly. The incidence of diarrhea in these patients is linked to the type of antibiotic

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### Table 14.1 Targets of Selected Probiotics for Elderly Subjects

<table>
<thead>
<tr>
<th>Target</th>
<th>Age (Years)</th>
<th>Strains</th>
<th>Results</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immune function</td>
<td></td>
<td><em>Bifidobacterium lactis</em> HN019</td>
<td>Increased natural killer cell and phagocytic activity</td>
<td>Chiang et al. (2000)</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Lactobacillus rhamnosus</em> HN001</td>
<td>Increased natural killer cell and phagocytic activity</td>
<td>Sheih et al. (2001)</td>
</tr>
<tr>
<td></td>
<td>84</td>
<td><em>Bifidobacterium longum</em> 2C and 46 and <em>B. lactis</em> Bb-12</td>
<td>Reduction of the inflammatory state</td>
<td>Ouwehand et al. (2008)</td>
</tr>
<tr>
<td></td>
<td>60–79</td>
<td><em>Bifidobacterium animalis</em> DN-173 010</td>
<td>Reduction on the duration of infections</td>
<td>Meance et al. (2001)</td>
</tr>
<tr>
<td>Bowel function</td>
<td>76–95</td>
<td><em>L. rhamnosus</em> and <em>Propionibacterium freudenreichii</em></td>
<td>Increased defecation frequency</td>
<td>Ouwehand et al. (2002)</td>
</tr>
<tr>
<td></td>
<td>61–102</td>
<td><em>B. longum</em> 46, <em>B. longum</em> 2C, and <em>B. lactis</em> Bb-12</td>
<td>Increased defecation frequency</td>
<td>Pitkälä et al. (2007)</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Lactobacillus acidophilus</em> and <em>Bifidobacterium bifidum</em></td>
<td>Incidence of <em>Clostridium difficile</em> toxins was lower in samples of feces of the treated group with probiotic</td>
<td>Plummer et al. (2004)</td>
</tr>
<tr>
<td>Diarrhea associated with the use of antibiotics</td>
<td>74</td>
<td><em>Lactobacillus casei</em>, <em>Lactobacillus bulgaricus</em>, and <em>Streptococcus thermophilus</em></td>
<td>Decreased morbidity</td>
<td>Hickson et al. (2007)</td>
</tr>
<tr>
<td></td>
<td>50–70</td>
<td><em>L. acidophilus</em>, <em>Lactobacillus paracasei</em> Lpc-37, <em>B. lactis</em> Bi-07, and <em>B. lactis</em> Bl-04</td>
<td>Decreased incidence of fever, abdominal pain, bloating, number of loose stools, and duration of diarrhea episodes</td>
<td>Ouwehand et al. (2014)</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Lactobacillus plantarum</em> 299v</td>
<td>Decreased colonization of <em>C. difficile</em></td>
<td>Klarin et al. (2008)</td>
</tr>
</tbody>
</table>
used and the health of the individual. Typically, about 15%–39% of cases are caused by *Clostridium difficile* and may result in pseudomembranous colitis and toxic megacolon (Allen et al., 2013). A meta-analysis by D’Souza et al. (2002) found that the organisms that have the greatest potential in this case are the *Lactobacillus* spp. and *Saccharomyces boulardii*.

Plummer et al. (2004) assessed the effect of supplementation of *Lactobacillus acidophilus* and *Bifidobacterium bifidum* on the incidence of diarrhea caused by *C. difficile* in elderly patients treated with antibiotics and found that the incidence of *C. difficile* toxins was lower in samples of feces of the group treated with probiotics (2.95%) compared to control (7.25%).

Hickson et al. (2007) observed a decreased morbidity in elderly patients diagnosed with *C. difficile*-associated diarrhea after consumption of a probiotic drink during the treatment period with antibiotics. Klarin et al. (2008) found that *Lactobacillus plantarum* 299v supplementation decreased the colonization of *C. difficile* in critical elderly patients submitted to treatment with antibiotics.

Ouwehand et al. (2014) reported that the combination of four strains of probiotics, when administered in high doses in patients diagnosed with diarrhea associated with antibiotic use caused by *C. difficile*, was able to decrease the incidence of fever, abdominal pain, and bloating as well as the number of loose stools and the duration of diarrhea episodes. The use of probiotics in these individuals has been shown to be a promising alternative in the prevention of diarrhea associated with antibiotic use, but more studies are needed.

Other studies have shown that some lactic acid-producing organisms possess antioxidant properties (Lin and Yen, 1999; Uskova and Kravchenko, 2008). The antioxidant mechanisms of probiotics can be attributed to reactive oxygen species’ scavenging capacity, metal ion chelation, and inhibition of auto-oxidation of ascorbate (Lin and Yen, 1999). It is known that oxidative stress plays a key role in the pathogenesis and progression of chronic diseases such as diabetes (Maritim et al., 2003). Studies in animal models of diabetes patients showed that administration of *L. acidophilus* and *L. casei* attenuated oxidative stress and disease symptoms (Yadav et al., 2007; Harisa et al., 2009). Ejtahed et al. (2012) observed that the consumption of a probiotic yogurt containing *L. acidophilus* La5 and *B. lactis* Bb-12 was able to improve the fasting glucose and antioxidant status of elderly and middle-aged patients with type 2 diabetes.

In the literature, the use of probiotics in elderly subjects has shown promise; however, more studies are necessary to better understand the influence of probiotics on elderly health.

### 14.3 Prebiotics on the Elderly Population

Prebiotics are defined as “selectively fermentable ingredients that allow specific changes in the composition and/or activity of the gastrointestinal microbiota that allow benefits to the host” (Gibson et al., 2004, 2010; Roberfroid et al., 2012). Prebiotics are naturally available in breast milk and in certain vegetables, although in low amounts, but can also be synthetic oligosaccharides (Toward et al., 2012). Nowadays, the most well-known prebiotics are nondigestible carbohydrates including fructooligosaccharides (FOS), inulin, galacto-oligosaccharides, and lactulose. Other nondigestible carbohydrates have been studied for their prebiotic potential; these include soybean oligosaccharides, isomaltooligosaccharides, xylooligosaccharides, polydextrose, glucans (Roberfroid, 2007; Gibson et al., 2010), and yacon (Almeida et al., 2015).
The effects of prebiotics are related to improvement and stabilization of the composition of the intestinal microbiota, improved bowel function (volume, consistency, and regularity of feces), increased mineral absorption (mainly calcium), modulation of immunological barriers of the intestine, or improvement of the immune system and improvement in the inflammatory reactions, reducing the risk of colon cancer (Roberfroid et al., 2012).

The composition and metabolic activities that occur in the gut contribute to the maintenance of health and the prevention of disease in individuals. The composition of the intestinal microbiota changes with the stages and ages of individuals. The gut of a baby is sterile, but when born it is rapidly colonized with bacteria from the mother. The development of the microbiota of the infant depends on the mother’s diet and the initial microbiota usually colonized are coliforms, enterococci, Lactobacillus and Bifidobacterium, which become predominant (Hattingh and Viljoen, 2001). When solid food is introduced in the diet of the newborn, the microbiota begins to diversify (Duncan and Flint, 2013). The microbiota of adults is well diversified and has around $10^{14}$ bacterial cells. In healthy individuals the predominant phyla are Firmicutes, Bacteroides, Actinobacteria, and fewer Proteobacteria (Nyangale et al., 2014).

In the elderly, the diversity of microbiota is reduced, decreasing the number of Bifidobacterium and increasing the number of Enterobacteriaceae, and certain Proteobacteria are suspected in some intestinal diseases (Duncan and Flint, 2013). In the microbiota of the elderly (>65 years) there is a reduction of Firmicutes, especially Bifidobacterium, and an increase in Proteobacteria, which has a variety of opportunistic pathogens (Nyangale et al., 2014).

In the elderly, the risk of disease is higher and many factors can contribute to changes in intestinal microbiota. Factors such as the use of medication and its side effects, and the deficiency of calcium intake and vitamins in the diet of the elderly can cause malnutrition and an imbalance in the functioning of the intestinal tract, such as constipation, increased risk of intestinal diseases, and fermentation of harmful products in the colon (Biagi et al., 2012; Duncan and Flint, 2013; Patel et al., 2014).

Studies in adult and elderly subjects showed that the use of prebiotics increased both the population of Bifidobacterium and the frequency of stools, and reduced the inflammatory processes (Patel et al., 2014).

While Bifidobacterium decline related to age can be reversed by prebiotics, clarification of the specific health outcomes of this organism is not fully apparent. Bifidobacterium is a recognized inhibitor of gut pathogens, so one beneficial property of increasing its numbers and activities is almost certain to be improved resistance to infections, and prebiotic activity has particular relevance for elderly persons (Toward et al., 2012).

The main products resulting from microbial metabolism of fiber are short-chain fatty acids (SCFAs), the concentrations of which in the colon are critically important for immunoregulation and for maintaining gut and overall health (Cuervo et al., 2013). The SCFAs produced by the fermentation of prebiotics have beneficial effects against cancer cells upon interaction with the intestinal microbiota to inhibit tumor cell growth and increase the enzymes responsible for detoxification (Patel et al., 2014). Cuervo et al. (2013) verified the association between food sources of fiber and fecal acetate, propionate, and butyrate concentration using a cross-sectional design study of the elderly. The results showed that potato intake was directly associated with SCFA concentrations and apple intake with propionate concentration. Of the fibers, cellulose showed an independent association with acetate and butyrate concentrations, and part of the variation in propionate may be caused by
insoluble pectin. These results provide further evidence regarding the relationship between diet and SCFA concentration in the elderly.

Prebiotics are also able to increase the absorption of calcium. With the increase in bacterial population and production of SCFAs, there is an increased substrate supply on the absorption surface, and consequently more fermentation products that bind the calcium and make it available for absorption (Patel et al., 2014).

There are a few results about the effects of probiotic or prebiotic yogurt on elderly health. Therefore, based on the available literature, it was possible to conclude that prebiotics consumption in yogurt or other products by the elderly population can improve bowel function (volume, consistency, and regularity of feces), increase mineral absorption (mainly calcium), increase modulation of immunological barriers of the intestine, increase SCFA production, improve the immune system, and improve the gut microbiota. However, more studies, especially clinical trials with prebiotic ingredients, are necessary to understand the specific mechanisms involved in the improvement of elderly health.

14.4 SYNBIOTIC YOGURT ON THE ELDERLY POPULATION

Although the elderly are a growing global segment, with an estimated growth of 300% for this population in Asia and America in the next 30 years, studies relating to the effects of the administration of synbiotic yogurts or synbiotic products on elderly health are still scarce (Amarya et al., 2015; Department of Economics and Social Affairs, 2012; National Sample Survey Organization, 2011).

The maintenance of health and life quality of elderly people is essential, thus the use of strategies that contribute to this situation are essential. The use of synbiotic products, such as a synbiotic yogurt, can positively contribute to the health of this population (Moroti et al., 2012).

Yogurt consumption has been associated with health benefits in different populations and yogurt is accessible and convenient to consume by the older population, which makes yogurt consumption a feasible approach to enhance older adults’ nutritional status (El-Abbadi et al., 2014). However, only a limited number of studies have specifically addressed the impact of synbiotic yogurt on the nutritional and health status of older adults.

The term “synbiotic” refers to the combined relationship between probiotics and prebiotics. This combination can contribute to the improvement in the survival of probiotics and provide additional health benefits to the host. In general, it has been widely used in food products to take advantage of their synergistic effects (Allgeyer et al., 2010; Al-Sheraji et al., 2013; Valero-Cases and Frutos, 2015). The addition of prebiotics to yogurts has also been related to the promotion of intestinal health and the improvement of the absorption of calcium and other minerals (Scholz-Ahrens et al., 2007; Gonzales et al., 2011).

The synbiotic interaction enhances the growth of beneficial bacteria in the organism and increases the production of SCFAs. The production of butyrate, propionate, and acetate provides energy to the gut microbiota and acidifies the intestinal lumen, creating an unfavorable environment for pathogenic bacteria. Furthermore, synbiotic products can reduce osteoporosis and contribute to the improvement of mineral absorption (Charalampopoulos et al., 2002; Scholz-Ahrens et al., 2007; Walsh et al., 2010).

Table 14.2 presents some clinical trials showing the effects of synbiotics on elderly individuals.

In general, the potential health effects of synbiotic yogurts on the elderly include changes in the composition and activity of the intestinal microbiota, especially by promoting growth of
SYNBIOTIC YOGURT ON THE ELDERLY POPULATION

Bifidobacterium and Lactobacillus in the gut; alleviation of constipation, increase of evacuation frequency, immunomodulation, decrease of cholesterol level, which may prevent and/or limit the effects of immunosenesence; and improvement of nutrient availability, especially regarding increased calcium and magnesium availability (Bedani et al., 2016).

During the aging period, changes occur in the gastrointestinal tract, decreasing the secretion of gastric acid, which interferes with the absorption of iron and vitamin B12. Saliva production is also decreased, retarding peristalsis (Amarya et al., 2015). Molina et al. (2012) developed a soybean-based functional food using Lactobacillus reuteri CRL 1098 and evaluated the efficiency of the biofortified soymilk obtained to prevent the symptoms produced by nutritional vitamin B12 deficiency in pregnant female mice, and their results showed that the administration of fermented soymilk prevented the development of all symptoms observed as a consequence of nutritional B12 deficiency in female mice. Vitamin B12 is exclusively synthesized by some bacteria and archaea (Martens et al., 2002), and according to Taranto et al. (2000) L. reuteri can synthesize vitamin B12.

Table 14.2 Clinical Trials Showing the Effects of Synbiotics on Elderly Individuals

<table>
<thead>
<tr>
<th>Study Design</th>
<th>Subjects (n; Age)</th>
<th>Product, Dose, and Period</th>
<th>Results</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Single treatment study</td>
<td>10 participants aged between 69 and 96</td>
<td>Commercial synbiotic consisting of fermented cow’s milk enriched with Lactobacillus rhamnosus Gorbach and Goldin (bottle of 10⁷ bacteria/90 g) and soluble fibers of oligofructose administered twice daily before breakfast and dinner for a period of 1 month</td>
<td>↑ Serum levels of IL-1β, IL-6, and IL-8</td>
<td>Amati et al. (2010)</td>
</tr>
<tr>
<td>Randomized, double-blind</td>
<td>20 volunteers aged between 50 and 65</td>
<td>200 mL of synbiotic shake containing 10⁸ cfu/mL of Lactobacillus acidophilus, 10⁸ cfu/mL of Bifidobacterium bifidum, and 2 g of oligofructose for 30 days</td>
<td>↑ Bifidobacterium spp. ↑ Lactobacillus spp. ↓ Clostridium spp. ↓ Bacteroides spp.</td>
<td>Moroti et al. (2010)</td>
</tr>
<tr>
<td>Randomized, double-blind</td>
<td>50 volunteers aged between 76 and 90. All with constipation problems</td>
<td>Yogurt containing 6.9 log cfu/g of Lactobacillus rhamnosus GG and 2.4% of fructooligosaccharides (FOS). 250 g of yogurt per day for 4 weeks</td>
<td>↑ Number of evacuations ↑ Bacteroides spp. ↑ Lactobacillus spp.</td>
<td>Granata et al. (2012)</td>
</tr>
<tr>
<td>Randomized, double-blind</td>
<td>20 women aged between 50 and 60 with total cholesterol &gt;200 mg/dL, triglycerides &gt;150 mg/dL, and blood glucose &gt;110 mg/dL</td>
<td>Symbiotic shake composed of 4 × 10⁸ cfu/100 mL of L. acidophilus, 4 × 10⁸ cfu/100 mL of B. bifidum, and 1 g/100 mL of FOS. 100 mL administered twice a day, totaling 200 mL/day for 30 consecutive days</td>
<td>↓ 25.84% of total cholesterol ↓ 37.27% of triglycerides ↓ 38.89% of blood glucose levels ↓ 35.15% of high-density lipoprotein levels</td>
<td>Moroti et al. (2012)</td>
</tr>
</tbody>
</table>
There is a reduction in bacterial biodiversity and an increase in colonization by pathogens during aging. Colonies of *Bifidobacterium* decrease, favoring clostridia, streptococci, and enterobacteria. The reduction in *Bifidobacterium* may adversely affect elderly health and one way to modulate the intestinal microbiota is the use of probiotics and prebiotics (Gibson and Roberfroid, 1995; Bartosch et al., 2004; Claesson et al., 2011; Granata et al., 2012; Nyangale et al., 2014). Moroti et al. (2010) observed a significant increase in *Lactobacillus* spp. and *Bifidobacterium* spp. in the microbiota of elderly people who consumed a shake containing synbiotics for 30 days (10⁸ cfu/mL of *L. acidophilus*, 10⁸ cfu/mL of *B. bifidum*, and 2 g of oligofructose).

Increasing age tends to be related to an increase in the administration of drugs, a modification of the diet, and a decrease in intestinal motility, thus causing a higher prevalence of constipation in the elderly. The reduction in food intake may therefore reduce the intake of fibers, important in preventing constipation. Intestinal motility may be changed because of the decrease in *Bifidobacterium* in the intestinal microbiota of healthy elderly. Thus the increased administration of prebiotics and probiotics can contribute to the reduction of constipation and relief of their symptoms (Hamilton-Miller, 2004; Woodmansey et al., 2004; Hsieh, 2005; Niittynen et al., 2007). Studies on the effects of daily administration of synbiotics on the elderly have shown an increase in the number of bowel movements, an increase in the colonies of *Bifidobacterium* and *Lactobacillus*, and a decrease in populations of *Clostridium* and *Bacteroides* (Moroti et al., 2010; Granata et al., 2012).

The natural aging process affects the immune system, causing a decline in immune function. A poor immune system contributes to the reduction of disease resistance and life expectancy (Sullivan and Lipschultz, 1997; Aspinall and Andrew, 2000; Nyangale et al., 2014; Amarya et al., 2015). Evidence for the influence of the microbiome on immunosenesence was provided by close correlations with markers of inflammation [serum levels of tumor necrosis factor, interleukin (IL)-6, IL-8, and C-reactive protein], which were notably increased in individuals in long-stay and rehabilitation care (Nicholson et al., 2012). Neto et al. (2013) investigated the influence of synbiotics on the inflammatory process in the elderly. The authors found no change in relation to inflammatory cytokines and no change in body composition.

Amati et al. (2010) indicated an increase in the serum levels of certain interleukins for seniors who made daily use of fermented milk synbiotics. In recent years, different studies have shown that the consumption of milk products, with or without the addition of probiotics and prebiotics, can have beneficial effects on the risk factors of the metabolic syndrome, including atherogenic dyslipidemia (Azadbakht et al., 2005), hyperglycemia (Perreira et al., 2002), insulin resistance (Rideout et al., 2013) or type 2 diabetes (Tong et al., 2011; Liu et al., 2006), blood pressure reduction (Toledo et al., 2009), and abdominal obesity (Wang et al., 2014). However, there are few studies that relate the consumption of probiotics and prebiotics dairy products to the health of older people. Moroti et al. (2012) found that daily consumption of a synbiotic shake by women over 50 years of age was able to reduce serum levels of cholesterol, blood sugar, and triglycerides, in addition to increasing the fraction of high-density lipoprotein cholesterol.

### 14.5 CONCLUDING REMARKS

In all countries the percentage of citizens aged 65 and over is increasing at an unprecedented rate, and is expected to account for over 30% of the population by 2050. It is necessary to find strategies to promote healthy aging. Adequate dietary intake is one of the key factors in maintaining good health and increasing the quality of life of the elderly. In the perspective of improving the health status of older people, the consumption of functional foods can open new ways to promote healthy aging.
As previously discussed, it is evident that probiotics, prebiotics, and synbiotics have the capacity to improve human health. Nevertheless, there are only a few results regarding the effect of synbiotic yogurt on elderly health. There are, however, indications that synbiotic yogurt has similar effects to other synbiotic products. The main action is related to the combination of probiotics and prebiotics. It is known that the effects of probiotics are dependent on the strain. In this context, the food matrix would be a carrier of probiotics and prebiotics, but some important food compounds can increase the synbiotic effect, such as bioactive compounds. It has been suggested in the literature that synbiotic products, including synbiotic yogurt, can improve bowel function (volume, consistency, and regularity of feces), increase mineral absorption (mainly calcium), increase the modulation of immunological barriers of the intestine, increase SCFAs, improve the immune system, decrease cholesterol level, and improve the gut microbiota. However, more studies are necessary, especially clinical trials with prebiotic ingredients, to better understand the specific mechanisms involved in the improvement of elderly health.

Finally, in the near future, as results of interactions of various fields of study become known and with technological advances in molecular analyses, the food market can offer products specifically for the promotion of healthy aging.

REFERENCES


**FURTHER READING**

PART

YOGURT AROUND THE WORLD 3
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15

NUNU, A WEST AFRICAN FERMENTED YOGURT-LIKE MILK PRODUCT

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15.1 INTRODUCTION

Nunu or nnono is the generic Hausa name for spontaneously fermented (yogurt-like) milk product in Ghana and other parts of West Africa. Nunu is prepared solely from cow’s milk. The same product or similar products with slight variations in processing methods may be known by different names depending on the local region or country of production. For example, nnu is the name used in northern Ghana, parts of Burkina Faso, and Nigeria, whereas other local names such as nyarmie, fènè, and lait caillé are used in southern Ghana (Obodai and Dodd, 2006), Mali (Wullschleger, 2009), and western Burkina Faso, respectively. In Côte d’Ivoire, the product is simply referred to as sour milk. Throughout the West African subregion, production of milk and fermented milk products is dominated by the nomadic Fulani tribesmen. Traditionally, men pastoralists oversee animal husbandry, whereas women are responsible for milk collection (Fig. 15.1), processing, and marketing (Akabanda, 2009).

15.2 PROCESSING OF WEST AFRICAN FERMENTED YOGURT-LIKE MILK PRODUCTS

Production of West African fermented yogurt-like milk products often depends on unique recipes and is handed down from generation to generation. Two main variations in the processing of West African yogurt-like milk products have been reported as described in Fig. 15.2. For production of nyarmie and fènè, milk from different cows is pooled together and sieved through an aluminum strainer or cheese cloth to remove particles of fur and other debris that may contaminate the freshly collected milk. The milk is then pasteurized by cooking in aluminum pots at 65–75°C for 30–50 min, cooled to room temperature, and the fat accumulating on the surface is collected (Obodai and Dodd, 2006; Wullschleger, 2009).

The process of pasteurization and defatting practiced in some communities in Ghana for nyarmie is similar to the production of fènè in Mali (Wullschleger, 2009). Except for the pasteurization and defatting steps, nnu production in Ghana, Burkina Faso, and Nigeria is similar to nyarmie and fènè (Fig. 15.2). The pasteurized or unpasteurized milk is kept in calabashes or plastic containers as shown in Fig. 15.3, covered with a lid, and allowed to spontaneously ferment at ambient temperature (28–35°C) for about 18–24 h. The natural fermentation results in the formation of curdled milk as shown in Fig. 15.4. Excess
CHAPTER 15  NUNU, A WEST AFRICAN YOGURT-LIKE MILK PRODUCT

FIGURE 15.1
Milking of cow’s milk for nunu production in northern Ghana by a Fulani woman.

*Courtesy of Fortune Akabanda, Ghana.*

FIGURE 15.2
Traditional processing of West African fermented yogurt-like milk products.
whey may be removed and then stirred vigorously or whipped with a wooden stirrer. This yields a set-style creamy and slightly sour yogurt-like product. The consistency of nunu varies from slightly smooth to a product with suspended milk curds. It may be viscous with a white to yellowish appearance (Akabanda et al., 2010, 2014; Obodai and Dodd, 2006; Wullschleger, 2009). Nunu can be consumed within 4–5 days after fermentation without refrigeration or refrigerated at 4°C for several weeks. It is often consumed alone as a beverage or blended with fura, a cereal-based fermented dough, and consumed as a complete
meal (Akabanda et al., 2013; Owusu-Kwarteng et al., 2012). The blend of nunu and fura into a meal is known as “fura de nunu.” Demand for nunu is usually high in the first quarter of the year and during the period of Ramadan, given its ability to quench thirst and refresh a sun-scorched and weary body. Unfortunately, the supply of raw milk for processing nunu and other dairy products is very low in the West African subregion, a problem compounded by the seasonality of the milk supply, which is dependent on rainfall patterns and availability of pasture.

15.3 MICROBIOLOGY OF WEST AFRICAN FERMENTED YOGURT-LIKE MILK PRODUCTS

Traditional milk fermentation is generally practiced in developing countries at home- or small-scale production by spontaneous fermentation. Thus no starter cultures are used in the fermentation of cow’s milk to produce West African yogurt-like products. The process of natural fermentation practiced in West African fermented yogurt-like products is one of the oldest methods of milk processing using raw and boiled milk to ferment spontaneously, or using the back-slopping method where a part of the previous batch of a fermented product is used to inoculate the new batch (Holzapfel, 2002; Josephsen et al., 2004). External factors such as regional or local climatic conditions (season, temperature), composition of the raw material, and the duration of the fermentation process influence the composition and activity of the microbial community associated with fermentation (Mathara et al., 2004), which in turn influence the unique organoleptic properties of the products (Steinkraus, 1994).

Recently, traditional fermentation of nunu in three smaller towns in northern Ghana was microbiologically characterized. Lactic acid bacterial counts increased during fermentation from an average of $4.5 \pm 0.4 \log$ cfu/mL at 0 h to $8.7 \pm 1.8 \log$ cfu/mL after 24 h, while yeasts increased from $2.8 \pm 1.2 \log$ cfu/mL at 0 h to $5.8 \pm 0.5 \log$ cfu/mL at the end of fermentation (Akabanda et al., 2013). Similarly, Obodai and Dodd (2006) isolated lactic acid bacteria (LAB) (grown on MRS agar) in the range of 7.11–9.03 log cfu/mL from nyarmie in southern Ghana. Furthermore, lactococci counts in nyarmie grown on M17 agar ranged between $10^7$ and $10^9$ cfu/mL after 48 h of fermentation, whereas the yeast count reached $10^7$ cfu/mL after 48 h of fermentation (Obodai and Dodd, 2006). In Malian fènè, lactic acid bacterial counts increased gradually throughout fermentation from 2 log cfu/mL at the beginning of fermentation to about 8 log cfu/mL after 22 h of fermentation. The initial high microbial load of raw milk of nearly $6 \log$ cfu/mL was partially but not completely reduced by the milk-heating step (Fig. 15.2) before fermentation under nonaseptic conditions. Presumptive enterococci were not detected on agar media until the fourth hour of fènè fermentation in Mali, but very high final counts at over $8 \log$ cfu/mL were detected (Wullschleger et al., 2013). Again, significant ($P < .05$) seasonal fluctuations of bacterial counts were observed at the end of spontaneous 22-h fermentation processes. Generally, lactic acid bacterial counts were significantly lower during the rainy and cold seasons than in the hot season in Mali. A reduction of counts on MRS agar from $9.2 \pm 0.5 \log$ cfu/mL in the hot season to $7.9 \pm 0.5 \log$ cfu/mL in the rainy season was observed (Wullschleger et al., 2013). In the Fulani traditional fermented milk in Burkina Faso, the mean count of mesophilic LAB isolated on MRS (35°C) reached $7.8 \times 10^8$ cfu/mL, whereas thermophilic LAB grown on MRS at 42°C reached $8.04 \times 10^5$ cfu/mL. Furthermore, lactococci counts reached $7.75 \times 10^7$ cfu/mL in Burkina Faso traditional yogurt (Savadogo et al., 2004a).

Microorganisms isolated from some common traditional fermented yogurt-like milk products in West Africa are shown in Table 15.1. Nunu fermentation in Ghana is dominated by LAB and yeasts
The taxonomic diversity of LAB and yeasts in nunu fermentation has been exploited using a combination of phenotypic and genotypic methods by Akabanda et al. (2013), which revealed that *Lactobacillus fermentum* dominates throughout the fermentation of nunu with *Lactobacillus plantarum* and *Leuconostoc mesenteroides* playing prominent roles during the first 6–8 h of fermentation as well. Less frequently isolated LAB included *Lactobacillus helveticus*, *Enterococcus faecium*, *Enterococcus italicus*, and *Weissella confusa*. The yeasts involved in nunu fermentation are *Candida parapsilosis*, *Candida rugosa*, *Candida tropicalis*, *Galactomyces geotrichum*, *Pichia kudriavzevii*, and *Saccharomyces cerevisiae* with *P. kudriavzevii* and *S. cerevisiae* being the dominant yeast species.

Similarly, the predominant microorganisms in nyarmie produced in southern Ghana include LAB and yeasts (Obodai and Dodd, 2006). During the characterization of microorganisms in fènè samples in Mali, Wullschleger et al. (2013) identified several species of LAB with enterococci and streptococci as dominant populations of the fènè microbiota. The conspicuous absence of *Streptococcus thermophilus*, usually occupying a central fermentative role, was further underscored by the finding that 87 streptococci selected for further typing were all found to be the putative human and animal pathogen *Streptococcus infantarius ssp. infantarius*. These findings suggested that spontaneously fermented fènè could be a reservoir for the potential

<table>
<thead>
<tr>
<th>Product Name</th>
<th>Country</th>
<th>Microorganisms</th>
<th>References</th>
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<tbody>
<tr>
<td>Nunu</td>
<td>Northern Ghana</td>
<td><em>Lactobacillus fermentum</em>, <em>Lactobacillus plantarum</em>, <em>Lactobacillus helveticus</em>, <em>Leuconostoc mesenteroides</em>, <em>Enterococcus faecium</em>, <em>Enterococcus italicus</em>, <em>Weissella confusa</em>, <em>Candida parapsilosis</em>, <em>Candida rugosa</em>, <em>Candida tropicalis</em>, <em>Galactomyces geotrichum</em>, <em>Pichia kudriavzevii</em>, <em>Saccharomyces cerevisiae</em></td>
<td>Akabanda et al. (2013)</td>
</tr>
<tr>
<td>Fermented milk</td>
<td>Burkina Faso</td>
<td><em>L. fermentum</em>, <em>L. mesenteroides</em> ssp. <em>mesenteroides</em>, <em>Lactobacillus acidophilus</em>, <em>S. thermophilus</em>, <em>Lactococcus spp.</em>, <em>Pediococcus spp.</em></td>
<td>Savadogo et al. (2004a,b,c)</td>
</tr>
<tr>
<td>Lait caillé</td>
<td>Western Burkina Faso</td>
<td>Unknown</td>
<td>Not applicable</td>
</tr>
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pathogenic strains of *S. infantarius* ssp. *infantarius* and as a likely source for human infection transmission. A similar observation was made for Ivorian fermented milk where about 44% of samples contained *S. infantarius* ssp. *infantarius* (Jans et al., 2013). *Listeria* spp. and *Listeria monocytogenes* have been associated with milk and milk products including nunu in Ghana (Alhassan, 2017; Appiah, 2012). These observations thus reinforce the need to develop starter cultures for the fermentation of the West African fermented yogurt-like milk products for improved health and safety.

As mentioned earlier, the production of West African yogurt-like products generally does not rely on the use of commercial starter cultures. Therefore stocks of previous ferment, fermentation containers, and environmental microorganisms contaminating the raw material are often used to initiate fermentation in new batches. The dependence on such an undefined and diverse microbial consortium during fermentations results in products of variable quality and stability. In an attempt to develop starter cultures for the fermentation of milk to produce nunu, LAB isolated from spontaneous nunu fermentation have been evaluated for various technological properties and tested as starter cultures for nunu production. The selected cultures, including *L. fermentum*, *L. plantarum*, *L. helveticus*, and *L. mesenteroides*, were used as single or combined starter cultures to produce nunu (Akabanda et al., 2014). *L. fermentum*, *L. helveticus*, and *L. plantarum* starter cultures, whether used singularly or in combination, produced West African yogurt (nunu) with desirable consumer sensory characteristics. However, further work on the use of such starters for the production of West African yogurt is needed for improved health and safety of consumers.

### 15.4 NUTRITIONAL COMPOSITION AND HEALTH BENEFITS

The chemical composition of a foodstuff provides a useful indication of its potential nutritional value. However, the composition of West African cow’s milk and yogurt-like products (nunu) have received little research attention.

Generally, milk is a major source of dietary energy, protein, and fat, contributing on an average 134 kcal of energy/capita per day, 8 g of protein/capita per day, and 7.3 g of fat/capita per day, respectively (STAT, 2012). However, when different geographic regions are considered, the contribution from milk to various nutritional components varies considerably. For example, milk provides only 3% of dietary energy supply in Africa compared with 8%–9% in Europe and Oceania; 6%–7% of dietary protein supply in Africa compared with 19% in Europe; and 6%–8% of dietary fat supply in Africa, compared with 11%–14% in Europe, Oceania, and the Americas (STAT, 2012).

Cow’s milk, the main raw material for nunu production in West Africa, generally contains (per 100 g of milk) 87.3–88.1 g of water, 3–4 g of fat, 3.2–3.4 g of total protein, 0.7 g of ash, 4.5–5.1 g of lactose, and 247–247 kJ of energy. Additionally, milk is considered to be an excellent source of essential amino acids for human nutrition, growth, and development (Kanwar et al., 2009). Milk protein has a high protein-digestibility-corrected amino acid score and the protein fraction contains peptides and other bioactive factors that may have specific effects on growth and recovery from undernutrition (Michaelsen et al., 2011). The average levels of major components of nunu are total solids (7.6%) of which 2.7% are proteins, fat (6.5%), ash content (0.6%), and carbohydrate (82.8%) (Egwaikhide et al., 2014). Nunu produced in Ghana by spontaneous fermentation and with various indigenous LAB as a starter has been shown to have varying levels of amino acids including essential
amino acids (Akabanda et al., 2014). For example, nunu produced by fermentation with indigenous starter culture of *L. plantarum*, *L. helveticus*, and a combination of *L. fermentum* and *L. plantarum* contained all eight essential amino acids including lysine, histidine, methionine, isoleucine, threonine, valine, phenylamine, and leucine at varying levels. On the other hand, the use of *L. fermentum* as a single starter culture-produced nunu had undetectable levels of valine and serine (Akabanda et al., 2014). Thus depending on the type of starter culture used for fermentation, nunu may have different nutritional or chemical compositions, which in turn may affect the health benefits derived from the product.

During production of nunu, the fermenting microorganisms interact with the milk components and transform the liquid milk into a yogurt-like product. These fermenting microorganisms may produce beneficial metabolites or may themselves interact with the host in a positive manner, referred to as the probiotic effect (Roupas et al., 2009; Stanton et al., 2005). LAB isolated from nunu in Ghana and Burkina Faso have been shown to produce exopolysaccharides (EPSs) in milk during fermentation (Akabanda et al., 2014; Savadogo et al., 2004c). EPSs produced by LAB not only impart highly desirable rheological changes in the food matrix such as increased viscosity and improved texture (Badel et al., 2011), but also could exert health beneficial effects through immunomodulation or by other means (Kumar et al., 2009; Rodríguez-Carvajal et al., 2008). The possibility of LAB EPSs acting as prebiotic substrates has previously been successfully demonstrated (Korakli et al., 2002). Therefore the use of indigenous EPSs producing LAB for nunu fermentation could impart positive health benefits to consumers. Thus EPSs synthesized by LAB may act as fermentable substrates for microorganisms in the human gut environment, modifying interactions among intestinal populations.

Throughout West Africa, consumption of nunu has traditionally been associated with series of health-promoting properties, but most of these health-promoting claims are yet to be confirmed scientifically. The nomadic Fulani tribe in West Africa consumes nunu for its purported health benefits aside from the nutritional benefits associated with consuming milk and its products. They believe that nunu offers protection against ailments such as diarrhea and constipation, but this has yet to be confirmed scientifically.

West African yogurt-like products are known to be better tolerated by people with lactose intolerance compared to raw milk, primarily because they contain less lactose (Panesar, 2011). In particular, yogurt containing live bacteria may be better tolerated by lactose malabsorbers because of the β-galactosidase in LAB used for making yogurt or the presence of bacteria in the yogurt that produce β-galactosidase in the small intestine. Furthermore, yogurt takes longer to pass through the digestive system than raw milk, thus allowing a more effective breakdown of lactose (Buttriss, 1997).

LAB in nunu have shown antimicrobial activity against foodborne pathogens. In Ghana, LAB cultures isolated from naturally fermented nunu have been shown to inhibit the growth of the human pathogens *Escherichia coli*, *L. monocytogenes*, *Salmonella typhi*, *Staphylococcus aureus*, and *Bacillus cereus* (Akabanda et al., 2014). Similarly, LAB isolated from yogurt-like products in Burkina Faso produced bacteriocin-like compounds, which exhibited antibacterial activities against *Enterococcus faecalis* 103907 CIP, *B. cereus* 13569 LMG, *S. aureus* ATCC 25293, and *E. coli* 105182 CIP (Savadogo et al., 2004b). However, further research is needed to purify and study in detail the characteristics of the antimicrobial substances produced by LAB isolated from nunu before their commercial applications.
15.5 CONCLUSION AND PERSPECTIVE

In this chapter, an overview of the production processes, microbiological characteristics, and composition and potential health benefits of West African fermented yogurt-like milk products has been presented. Undoubtedly, nunu is one of the less characterized fermented yogurt-like products in Africa. Although it is difficult to reach a firm conclusion about the health impact of nunu, in general it can be consumed as part of a healthy, balanced diet, provided that the raw milk for processing is pasteurized and strict hygienic practices are observed during processing. The production and consumption of nunu can play a significant role in promoting lifelong healthy lifestyles and in alleviating protein energy malnutrition in infants by serving as a source of minerals, vitamins, essential amino acids, and probiotics, especially in the poor communities of West Africa. This can be achieved by orientating research to produce nutrient-rich and healthy dairy products (nunu) and supporting the science that will fill the existing knowledge gaps. Research geared toward the development of starter culture(s) for the production of nunu will ensure consumer safety and consistent quality of the final product.

REFERENCES


TRADITIONAL YOGURT AS A SOURCE OF LACTOBACILLI AND OTHER LACTIC ACID BACTERIA IN IRAN

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16.1 INTRODUCTION

Yogurt is one of the most consumed and most popular dairy products in Iran. Traditional dairy products have been used for many centuries by the Iranian people and tribes (Jafari et al., 2011). Evidence suggests that milk was cultured and fermented as early as 2000 BC in the Iranian-Indo cultures. Yogurt was used not only as a food but also as an irreplaceable medicine (Nikkah, 2014). After the Iranian people embraced Islam, Islamic regulations and guidelines affected their nutrition. In Islamic traditions, there is an emphasis on the use of dairy products, and consumption of yogurt in combination with some ameliorating compounds has been recommended (Mohammadi Rey Shahri, 2004).

In recent years, consumption of food products containing probiotic microorganisms has increased among Iranians (Haghshenas et al., 2016). In Iran, yogurt may be consumed by itself or mixed with herbs like mint or and fruits and vegetables such as cucumber. Besides yogurt, dough as a nonalcoholic fermented drink derived from diluted milk or yogurt is one of the favorite drinks among Iranians. It is usually served after meals. Interestingly, according to available raw materials (milk or yogurt) and traditional production processes, different kinds of dough are produced in Iran. One of the routine methods for its production is diluting traditional yogurt with water. To enhance the taste, some additives such as salt, pepper, and mint may also be added.

A lot of research has been carried out over the past three decades about the isolation of lactic acid bacteria (LAB) from Iranian dairy products especially milk, yogurt, and about the health benefits of different kinds of traditional and conventional (ordinary or probiotic) yogurt (Baroutkoub et al., 2010; Ejtahed et al., 2011).

The aims of microbial isolation from the Iranian dairy products have been either to investigate their microbial flora or to use the strains as a starter culture for yogurt production (RoushanZadeh et al., 2014). In addition, some of the studies have used the isolates as local probiotic strains, while others have examined health benefits of Iranian dairy products containing probiotics.

In this chapter, research works about the isolation of different LAB from Iranian traditional yogurt or dough have been highlighted. In addition, important relevant studies about their potential health benefits have been briefly highlighted.


16.2 LACTOBACILLI

16.2.1 LACTOBACILLUS ACIDOPHILUS

The isolation of Lactobacillus acidophilus from yogurt samples from five states in Iran in 1996 was reported by Nakhdjavani et al. Similarly, its isolation has been also reported by other researchers. In 2010, Izadi et al. also isolated L. acidophilus from yogurt samples of Shahr Babak. In another study, RoushanZadeh et al. (2014) collected different samples of yogurt from four different geographical regions of Fars, Khoozestan, Kerman, Yazd, Khorasan, Shahre Kord, Gilan, and Mazandaran. They also isolated L. acidophilus (RoushanZadeh et al., 2014). In addition, Rashti et al. isolated L. acidophilus from dough samples from Gorgan city in 2015 (Rashti et al., 2015).

16.2.2 LACTOBACILLUS BREVIS

The isolation of Lactobacillus brevis from yogurt and cheese samples in 2010 was reported by Ebrahimi et al. (2011). In 2014, Iranmanesh et al. and RoushanZade et al. separately reported its isolation from yogurt of different parts of Iran. Recently, the isolation of L. brevis from yogurt and milk samples of different areas of the Khorasan-e-Razavi was reported.

16.2.3 LACTOBACILLUS CASEI

According to the authors’ knowledge, Lactobacillus casei was first isolated from yogurt samples of five states of Iran in 1996 by Nakhdjavani et al., and then, its isolation was reported by Rashti et al. in 2010 from yogurt and cheese samples (Ebrahimi et al., 2011). Emami et al. (2013) reported the isolation of L. casei from traditional yogurt of Isfahan state in 2014. Then in 2015 L. casei was isolated from dough samples of Gorgan city by Rashti and Koohsari.

16.2.4 LACTOBACILLUS DELBRUECKII

Lactobacillus delbrueckii has been isolated from yogurt of different regions of Iran. In 1996, the isolation of Lactobacillus bulgaricus (later renamed Lactobacillus delbrueckii ssp. bulgaricus) from yogurt samples of five states of Iran was reported by Nakhdjavani et al. Then, Bonyadi et al. in 2011 isolated L. delbrueckii from yogurt samples of villages of East-Azerbaijan. In 2014, RoushanZadeh et al. reported the isolation of L. delbrueckii from yogurt samples of four geographical regions of Fars, Khoozestan, Kerman, Yazd, Khorasan, Shahre Kord, Gilan, and Mazandaran. Then, in 2015, L. delbrueckii was isolated from dough samples of Gorgan city (Rashti and Koohsari, 2015). After that, isolation of L. delbrueckii ssp. bulgaricus and Lactobacillus delbrueckii ssp. lactis were reported by Hajimohammadi Farimani et al. in 2016 from yogurt and milk samples of different areas of the Khorasan-e-Razavi region.

16.2.5 LACTOBACILLUS FERMENTUM

RoushanZadeh et al. in 2014 reported the isolation of Lactobacillus fermentum from yogurt samples of four geographical regions of Fars, Khoozestan, Kerman, Yazd, Khorasan, Shahre Kord, Gilan and Mazandaran.
16.2.6 *Lactobacillus helveticus*

The isolation of *Lactobacillus helveticus* from yogurt samples was first reported in 1996 by Nakhdjavani et al. They isolated this organism from different yogurt samples collected from five states of Iran (Nakhdjavani et al., 1996). However, it was also isolated later by others. RoushanZadeh et al. in 2014 reported the isolation of *L. helveticus* from yogurt samples of four geographical regions of Iran. Recently, Farimani et al. (2016) isolated and identified *L. helveticus* from yogurt and milk samples of different areas of the Khorasan-e-Razavi region.

16.2.7 *Lactobacillus lactis*

The isolation of *L. lactis* from yogurt samples of five states of Iran in 1996 was reported by Nakhdjavani et al.

16.2.8 *Lactobacillus paracasei*

Haghshenas et al. in 2016 reported the isolation of *Lactobacillus paracasei* ssp. *paracasei* from yogurt samples of rural areas of Kermanshah.

16.2.9 *Lactobacillus pentosus*

Emami et al. (2013) reported the isolation of *Lactobacillus pentosus* from traditional yogurt of Isfahan state in 2014. The isolation of *L. pentosus* from ewe’s milk and traditional yogurt samples of Azarbajjan-e-Sharqi state, in the northwest of Iran, in 2014 was reported by Iranmanesh et al.

16.2.10 *Lactobacillus plantarum*

Tajabadi Ebrahimi et al. in 2010 isolated *L. plantarum* from yogurt and cheese samples (Ebrahimi et al., 2011). RoushanZadeh et al. in 2014 reported the isolation of *L. plantarum* from yogurt and milk samples of four geographical regions of Iran. Emami et al. (2013) reported the isolation of *L. plantarum* from traditional yogurt of Isfahan state in 2014. Recently Hajimohammadi Farimani et al. (2016) isolated and identified *L. plantarum* from yogurt and milk samples of different areas of the Khorasan-e-Razavi region.

16.2.11 *Lactobacillus rhamnosus*

The isolation of *Lactobacillus rhamnosus* from yogurt samples of five states of Iran in 1996 was reported by Nakhdjavani et al.

16.2.12 *Lactobacillus spp.*

There are also some other reports about isolation of *Lactobacillus* from yogurt in Iran, but the species of isolated *Lactobacillus* have not been identified (Heshmatipour et al., 2015).
16.3 STREPTOCOCCI

According to the authors’ knowledge, a few studies have successfully isolated *Streptococcus* spp. from Iranian dairy products. It appears that appropriate culture media or physicochemical conditions were not used for isolation of this genus.

In 1996, Nakhjavani et al., isolated *Streptococcus lactis, Streptococcus diacetylactis, Streptococcus thermophilus, and Streptococcus fecalis* from Iranian dairy products including yogurt, cheese, and milk; however, they did not indicate the origins of dairy products. In addition, according to their report, streptococci were mostly isolated from cheese (Nakhdjavani et al., 1996). Roshanzadeh et al. isolated and identified different LAB bacteria from 60 yogurt samples collected from the tribes of four geographical regions of Iran. On the basis of their reports, among the 137 isolates that were identified as LAB on the basis of 16s rRNA, a minor percentage belongs to *S. thermophilus* and *Streptococcus acidominimus* (RoushanZadeh et al., 2014).

16.4 LEUCONOSTOC

In 1992, Nakhjavani et al. reported the isolation of *Leuconostoc mesenteroides* ssp. *cremoris* from yogurt. They collected 71 samples of milk and dairy products from 18 cities chosen from five states throughout Iran, but they have not reported the names of the cities (Nakhdjavani et al., 1996).

The isolation of *L. mesenteroides* ssp. *cremoris* from traditional drinking yogurt in tribes of Fars province was reported by Azadnia and Khan Nazer in 2009. Morphological, cultural, physiological, and biochemical characteristics were employed by them to identify LAB, isolated from drinking yogurt in different areas in Fars. From 18 drinking yogurt samples, a total of 673 LAB isolates were determined, of which 117 (17.38%) were identified as lactic acid cocci. Additionally, they showed the occurrence of 65 (55.56%) *L. mesenteroides* ssp. *cremoris* as dominant species among lactic acid cocci (Azadnia and Khan Nazer, 2009).

16.5 ENTEROCOCCI

Eslami (2008) isolated *Enterococcus durans, Enterococcus faecium, and Enterococcus faecalis* from traditional yogurt and cheese of Kleibar, East Azarbayegan. They characterized those isolates using phenotypic methods and genotyping with RFLP-16s rDNA gene, sequencing and RAPD-PCR analysis. They evaluated probiotic potential of the isolates and *E. faecium* expressed the best probiotic characteristics (Eslami, 2008).

Latifi et al. (2010) reported the isolation of 16 enterococci isolates from traditional cheese and yogurt of Heris and Sarab regions, East Azarbayegan, Iran. They isolated and characterized those isolates using phenotypic methods (Gram staining, physiological and biochemical tests). Then their acid and bile tolerances, as the primary probiotic characteristics, were investigated. The 16s rRNA gene of enterococci was amplified for identification of bacterial strains. Genotyping of isolates was performed by RAPD-PCR analysis using four random primers (Latifi et al., 2010).
Jafari et al. (2011) reported the isolation and identification of 26 Enterococcus isolates from four groups including E. durans, E. faecium, E. faecalis, and Enterococcus avium from traditional dairy products (yogurt, cheese, grout, and shour) of Ardabil region in Iran. They evaluated antimicrobial activity of four different Enterococcus isolates in pH 4 (culture pH) and 6.5 (neutralized pH) against three pathogenic bacteria including Escherichia coli strain PTCC 1399, Yersinia enterocolitica ATCC 1159 and Listeria innocua DSMZ 20649 by agar well diffusion method. All of the tested isolates showed inhibitory zones against pathogenic bacteria in pH 4, but two Enterococcus expressed inhibitory zones in pH 6.5 (Jafari et al., 2011).

Narimani et al. (2013) reported the isolation and identification of six strains of Enterococci from traditional yogurt of Kleibar and Varzaghan, East Azarbayegan, Iran, by culture and identified isolates based on biochemical properties and their resistance to stomach acid and bile salts.

RoushanZadeh et al. (2014) evaluated phenotypic and genotypic diversity of dominant lactic acid bacteria isolated from traditional yogurts produced by tribes of Iran. From 60 yogurt samples, a total of 137 LAB isolates were determined, of which 66 were identified as lactic acid cocci. Genetically, the presence of the following species was verified: E. faecium; E. faecalis; E. durans (RoushanZadeh et al., 2014).

16.6 LACTOCOCCI
The isolation of L. lactis ssp. cremoris from traditional drinking yogurt from the tribes of Fars province was reported by Azadnia and Khan Nazer in 2009. They used morphological, cultural, physiological, and biochemical characteristics to identify the organism (Azadnia and Khan Nazer, 2009).

The same methods were employed to identify lactococci isolated from yogurt in different areas in Kazerun city of Fars province in Iran by Azadnia et al. (2011). From 15 yogurt samples, a total of 28 lactococci were determined. Additionally, the biochemical tests and API kit showed that all of them were L. lactis ssp. cremoris (Azadnia et al., 2011).

16.7 PEDIOCOCCI
Soltan Dallal et al. (2015) studied 96 yogurt samples (from goat’s, sheep’s, and cow’s milk) collected from different parts of Yazd province. Lactic acid bacteria with probiotic characteristics were identified with biochemical tests and their probiotic activity was investigated by means of resistance to acid and bile. In general, out of 75 positive samples, 47 were identified as lactic acid bacteria on the bases of phenotype and biochemical tests, in which 24 were resistant to acid and out of these, 12 were resistant to bile. Five of them were identified as Pediococcus acidilactici, and isolated from sheep and goat yogurt (Soltan Dallal et al., 2015).

16.8 MAIN PURPOSES FOR CONSUMPTION OF TRADITIONAL YOGURT AS AN IMPORTANT SOURCE OF LAB IN IRAN
Despite the diversity of the studies about isolation of LAB from yogurt in Iran, the main purposes of their consumption can be summarized as follows.
16.8.1 ACHIEVING BETTER DAIRY STARTERS

Various kinds of yogurt that could be a source of valuable starter bacteria with different organoleptic properties are traditionally produced in Iran (RoushanZadeh et al., 2014). Interest in the microbiota of traditional fermented dairy products, such as yogurt, continues due to the need for new lactic acid bacteria strains that can complement or replace currently used starters (Farimani et al., 2016). The varied climate in Iran makes the production of a wide range of dairy products possible, particularly in rural regions. The Iranian consumer prefers to use traditional dairy products due to their excellent natural tastes and flavors. In this perspective, such products are potentially good candidates for isolating new strains of lactobacilli and other lactic acid bacteria (Ebrahimi et al., 2011). Some of the new isolated bacteria may have valuable characteristics of starter culture for yogurt production (RoushanZadeh et al., 2014). For example, among the 102 isolates from five traditional yogurts and one milk sample from different areas of the Khorasan-e-Razavi region, the thermophilic LAB species *S. thermophilus* and *L. delbrueckii* were the majority of bacteria in all samples (Farimani et al., 2016). The safety and technological features of 29 strains belonging to the species *S. thermophilus*, *L. delbrueckii*, and *L. helveticus*, all candidates for use in yogurt-making, were examined (Farimani et al., 2016).

16.8.2 PRODUCTION OF FOOD PRODUCTS CONTAINING PROBIOTIC MICROORGANISMS

In recent years, consumption of food products containing probiotic microorganisms has increased among Iranians (Haghshenas et al., 2016). Some of the studies have used local probiotic strains while others have examined the health benefits of Iranian dairy products containing probiotics. In a study by Haghshenas et al. (2016), a total of 19 LAB isolates were isolated from different dairy products including yogurt, shiraz (a kind of food derived from boiled yogurt), cheese, curd, and tarkhineh. Curd is a dairy product obtained by coagulating milk in a process called curdling (Wikipedia, 2016a). The coagulation can be caused by adding rennet or any edible acidic substance such as lemon juice or vinegar, and then allowing it to stand, and tarkhineh is a dried food based on a fermented mixture of grain and yogurt or fermented milk (Wikipedia, 2016b). All of them originated from a rural part of Kermanshah. Different genera of LAB were identified by sequencing of their 16s rRNA genes. *L. paracasei* ssp. *Paracasei*, which was isolated from yogurt, showed high survival rates under low pH (>70%) and high bile conditions (>87%). However, it could not resist against a simulated digestion test (Haghshenas et al., 2016). *L. plantarum* 15HL isolated from shiraz displayed ≥71% survival rates at low pH/high bile salts. It was also one of the most resistant strains to resist digestion conditions (≥40% survival rates). In addition, *L. plantarum* 15HN was the most adherent strain to Caco-2 cells. Furthermore, it exhibited high values of antiadhesion capability (≥36%). According to the mentioned results and some other tests, it has been concluded that it can be introduced as novel probiotic candidate that could be used in the food industry (Haghshenas et al., 2016).

16.8.3 INVESTIGATION OF ANTAGONISTIC EFFECTS OF ISOLATED LACTIC ACID BACTERIA

A series of studies have investigated the ability of isolated LAB sp. especially lactobacilli of traditional Iranian dairy products to inhibit pathogenic bacteria (Ebrahimi et al., 2011; Haghshenas et al., 2016; Heshmatipour et al., 2015; Rashti and Koohsari, 2015; Zare Javid et al., 2015).
In a study by Heshmatipour et al., lactic acid bacteria were isolated from native yogurt in the north of Iran. The isolated LAB were identified as *Lactobacillus* spp. Then, the antimicrobial activity of cell-free supernatant and partially purified bacteriocin-like inhibitory substance was determined by well diffusion method. The cell-free supernatant and partially purified bacteriocin-like inhibitory substance exhibited an antibacterial effect on a narrow range of extended spectrum β-lactamases (ESBLs) *E. coli* (Heshmatipour et al., 2015).

To assess *Lactobacillus* strains with high-quality probiotic potentials from different kinds of yogurts made by traditional dairy producers of Isfahan, Emami et al. (2013) isolated *Lactobacillus* strains from various traditional yogurts. Then, they determined probiotic properties of the selected lactobacilli. Strong acid and bile salt tolerant strains were considered as high-quality probiotics, identified at the species level by biochemical tests, and further identified according to 16s rRNA specific sequences. A total of 82 *Lactobacillus* strains were isolated. Fourteen strains were graded as high-quality probiotic lactobacilli (strong acid and bile tolerant). The phenotypic characterization and 16s rRNA gene sequencing assay of high-quality potentially probiotics strains resulted in identification of different *Lactobacillus* species including three *L. casei*, eight *L. plantarum*, and three *L. pentosus*. The results of antimicrobial potential of strong acid and bile tolerant strains against indicator bacteria demonstrated variable inhibitory activities. The majority of strains showed strong inhibitory potential against *E. coli*, *S. aureus*, and *Salmonella typhi*, but only two isolates (*L. casei* and *L. plantarum*) could inhibit *Pseudomonas aeruginosa* growth (Emami et al., 2013).

Besides yogurt, lactic acid bacteria were isolated from dough of different areas in Gorgan city. From 13 traditional dough and 2 industrial dough samples, a total of 35 isolates were isolated, 32 isolates from local dough and 3 isolates from industrial dough. The highest frequency of lactic acid bacteria belonged to *L. casei*, *L. acidophilus*, and *L. delbrueckii*, respectively. Also, these species showed antagonistic activity against pathogens including *E. coli*, *S. aureus*, *Bacillus cereus*, and *Citrobacter freundii* (Rashti and Koohsari, 2015).

16.9.1 DIABETES MELLITUS

The incidence of type 2 diabetes mellitus (T2DM) has rapidly increased in the world during the past few decades (Ejtahed et al., 2012), therefore a lot of studies have been carried out to prevent or treat it by modifying food regimes. In a clinical trial in Tehran, consumption of yogurt containing probiotics (*L. acidophilus* La5 and *Bifidobacterium lactis* Bb12) has been shown to decrease fasting blood glucose and hemoglobin A1c and increase erythrocyte superoxide dismutase and glutathione peroxidase activities and total antioxidant status. In addition, by consumption of the probiotic yogurt, the serum malondialdehyde concentration significantly decreased (Ejtahed et al., 2012). The results of this clinical trial suggest that probiotic yogurt is a promising agent for diabetes management (Ejtahed et al., 2012).

On the other hand, cardiovascular disease (CVD) is the primary cause of death in people with type 2 diabetes mellitus (T2DM) (Ejtahed et al., 2011). In a study in Tabriz, in the northwest of Iran, 60 people with type 2 diabetes and low-density lipoprotein cholesterol (LDL-C) greater than 2.6 mmol/L, consumed daily 300 g of probiotic yogurt containing *L. acidophilus* La5 and *B. lactis* Bb12 or 300 g of
conventional yogurt for 6 weeks. The results of the study showed that probiotic yogurt improved total cholesterol and LDL-C concentrations in type 2 diabetic people and may contribute to the improvement of cardiovascular disease risk factors (Ejtahed et al., 2011).

### 16.9.2 Cardiovascular Disease

Unfortunately, about 4% of the deaths in Iran are due to cardiovascular diseases, and hypercholesterolemia is among the aggravating factors (Baroutkoub et al., 2010). To assess the ability of LAB isolates to remove cholesterol from culture media, different dairy products including ewe’s milk, sour buttermilk and traditional yogurt, cheese, fermented milk, dough, and kashk (strained yogurt is cooked to make a sour cream sauce called kashk) (Wikipedia, 2016a,b) have been analyzed (Ebrahimi et al., 2011; Iranmanesh et al., 2014). In a study, 66 isolates of Lactobacillus spp. were obtained (Ebrahimi et al., 2011). The isolates were screened on the basis of their acid and bile tolerance and in vitro cholesterol removal. They exhibited both high cholesterol assimilation and antagonist activity. Interestingly, the levels of cholesterol assimilation during 24 h of growth varied considerably among the tested isolates and ranged from 34 to 276 μg/mL. Eleven isolates assimilated more than 50% of the cholesterol in the medium and seven isolates including L. casei y2c4, Lactobacillus farieminis C4i2, L. acidophilus Y2b9, Lactobacillus alimentarium Y114, L. plantarum C6m3, and L. plantarum C5i4 assimilated more than 75% cholesterol compared with the control (Ebrahimi et al., 2011).

In this respect, in another project, the ability of viable and dead cells of isolates that originated from traditional dairy products in Azarbayjan-e-sharqi to remove cholesterol was investigated. Four of the isolates were identified as L. brevis, L. pentosus, Pediococcus acidilactici and L. paracasei. In accordance with previous studies, it was revealed that even different isolates of same species exhibited different levels of cholesterol-lowering effects (Iranmanesh et al., 2014). L. brevis assimilated the highest level of cholesterol in viable cells during 2, 4, and 24 h. Compared to viable cells, the ability of the heat-killed cells to reduce cholesterol although present, but was significantly lower (Iranmanesh et al., 2014). These results are in agreement with the results of another study conducted in Iran, which showed significant decrease in serum cholesterol levels by consumption of probiotic yogurt in comparison with ordinary yogurt (Ataie-Jafari et al., 2009; Baroutkoub et al., 2010). For example, in a study in Shiraz, southern Iran, in a comparison between probiotic yogurt (containing L. acidophilus and Bifidobacterium) and ordinary yogurt, it was shown that consumption of probiotic yogurt for a period of 6 weeks reduced the total cholesterol and LDL significantly (Baroutkoub et al., 2010).

### 16.9.3 Diarrhea

Diarrhea is a well-recognized side effect associated with pelvic radiotherapy. The change in the intestinal bacterial flora, increased permeability of mucous cells and intestine and bowel movements may cause radiation-induced diarrhea. Mansouri-Tehrani et al. (2016) used clinical trials to show that probiotics with yogurt reduced the incidence of radiation-induced diarrhea and the need for antidiarrheal medication and had significant benefits on stool consistency in cancer patients.
16.10 CONCLUSIONS

Traditional yogurt in Iran is a very valuable source for isolation of lactic acid bacteria. Therefore, it is necessary to develop additional quantitative and qualitative research about isolation and identification of new LAB isolates from traditional yogurt to find the best dairy starters and efficient and stable probiotic microorganisms.

REFERENCES


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RHEOLOGY AND FUNCTIONALITY OF AYRAN—A YOGURT DRINK

17

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17.1 INTRODUCTION

Typical food dispersions generally consist of a mixture of lipids, proteins, carbohydrates, and water as an aqueous continuous phase. This continuous phase characterizes the mechanical properties. Prediction models dependent on compositions of such products are presently mostly based on thermal properties but not rheological characteristics (Herrmann et al., 2013). However, the composition and concentration of these constituents affect the rheological properties (Genovese et al., 2007; Mandala et al., 2004). Food dispersions can be either emulsion or suspension depending on the state of aggregation of the discontinuous phase. Emulsions are defined as a fine dispersion of one liquid in a second immiscible liquid (Grace, 2004). Suspensions are fluids that consist of a solid discontinuous phase and a continuous aqueous phase (Herrmann et al., 2013). These systems consist of hydrophilic (carbohydrates and some proteins, some of which amphiphilic) and hydrophobic parts (fats) (Sheldrake, 2003). The mechanical properties can be influenced by the amount, distribution, and stabilization of these parts (Herrmann et al., 2013). The increasing amount of fat results in increased viscosity (Sheldrake, 2003), mouthfeel, and satiety. Carbohydrates in the form of polysaccharides contribute to the structure and texture by bulking and formation of networks (Sheldrake, 2003). This contribution may help stabilization of an emulsion. Proteins function as emulsifiers with stabilization and sometimes thickening properties (Herrmann et al., 2013).

An understanding of structure–function and/or property relations of individual components in a mixed system containing protein and polysaccharide is of particular interest for creating functional ingredients in foods (Sharma et al., 2011). This approach may be extended to the understanding of relations between the structural and functional characteristics of the fermented drinks. For instance, both ayran and yogurt are made from milk, which is a Newtonian fluid (Fig. 17.1). The same starter cultures in both products are used for lactic acid fermentation. During yogurt fermentation, milk as a liquid turns into yogurt, which is a three-dimensional viscoelastic gel; somewhere in between, ayran (both contain suspension and emulsion structures in their body based on the previous definitions) can be obtained as a pseudoplastic or thixotropic fluid depending on its fat content. Pseudoplasticity, thixotropy, and viscoelasticity are very different rheological behaviors; their flow characteristics are different as well as their perceptions in the mouth (Altay et al., 2013). The rheological properties, which are influenced by structure and composition, may affect the functional properties in the body as well. If the structure–function relations for the fermentation process were to be constituted for fermented drinks, then it can be determined at which point and under what conditions these transitions take place.
Additionally, the stability of these drinks, their perception in the mouth, even their functionality in the body, may be tailored as needed.

Most of the studies related to fermented foods have focused on the fermentation process in terms of microbiota. On the other hand, the studies on the quality characteristics of fermented foods have been devoted to the determination of composition, color characteristics, rheological parameters, and sensory properties. In these studies, researchers have suggested composition-related solutions to the quality-related problems of fermented beverages (Altay et al., 2013). For instance, Dogan (2011) suggested that since maintaining texture in the commercial manufacture of fermented dairy products and keeping the quality of the final product can be problems, increasing milk solids may solve the textural problem. In another case, weak aroma and taste, low viscosity, sedimentation, and serum separation are the main problems, especially in semi-skimmed and skimmed ayran. The general trends in industrial applications and scientific studies for solving these problems include the addition of stabilizers and gums such as whey, locust bean gum, high...
methoxyl pectin, gelatin, and guar gum (Bayraktaroglu and Obuz, 2008; Köksoy and Kilic, 2004). In addition, utilization of transglutaminase for the crosslinking of proteins, which are considered the responsible molecules for texture in ayran, was reported for obtaining samples with higher viscosity and lower serum separation (Sanli et al., 2011). However, it appears that the effects of fermentation conditions such as substrate, time, temperature, microbiota, and duration on the structure of fermented products have not been studied. This is mainly because of the finishing/termination of the fermentation based on the general sensory properties and/or acidity of the fermented foods. Actually, the fermentation process is responsible for the structure of the product; this is ignored in most cases. Therefore, as a starting point, studies related to exopolysaccharide production starter cultures can be taken into account (Altay et al., 2013).

There has been a trend in the production of functional yogurt or fermented milk drinks recently. To make these drinks more functional, additives are included in the formula depending on the desired “function” such as easily digestible, healthier (meaning higher bioavailability/bioaccessibility), or having more antioxidant activity. When making the drinks more “functional” the researchers also study parameters such as palatability, aroma, acceptance, and perception in the mouth; these are related to rheological, textural, and sensory characteristics. Sometimes when a product is made more functional, it may have poor sensory properties, or vice versa. In this case, the same problem exists: studies on the functionality of the products are not really interested in structural characteristics and the causative factors of these characteristics (such as composition, concentration, and fermentation). If the structure–function relations have been taken into account for synthetic polymers, and it makes sense, then the same structure–function approach may be used for functional yogurt or fermented milk drinks. The factors and chemistry behind the rheological properties (which can also be related to sensory properties) during formulation, fermentation, and manufacturing of yogurt and fermented milk drinks may help to understand the functionality of the product. The key point of acceptance of any food (including appearance, mouthfeel, stability, aroma, and flavor) may be somewhat related to its bioavailability. In this context, the literature on functionality and rheological properties of yogurt or fermented milk drinks is summarized in this review.

17.2 YOGURT

Yogurt is the most popular fermented milk product worldwide and originated from countries around the Balkans and eastern Mediterranean Sea (Jaros and Rohm, 2003). Of all the cultured products, yogurt is probably the best known and most popular and is produced in various forms: as a viscous liquid, soft gel, drink, or in a frozen dessert (Scott, 1989).

Milk is a complex fluid containing relatively high amounts of proteins and minerals. The major part of milk proteins, the casein, which occurs in conjunction with calcium phosphate in the form of colloidal particles with 100–500 nm diameter and approximately $10^8 \text{Da}$, is of great importance for the functional behavior of the final acidified product. Colloidal calcium phosphate (CCP) plays an important role in maintaining the integrity of the casein micelles, which are in dynamic equilibrium with their surroundings. During fermentation of milk to yogurt, milk sugar in the base milk is partially converted into lactic acid by the action of various enzymes, originating from the growth of thermophilic lactic acid bacteria. This causes a sufficient decrease in the pH, resulting in a dissociation of the CCP, a destabilization of the casein micelles, further liberation of individual casein molecules (Jaros and Rohm,
2003; Routray et al., 2011), and maximum voluminosity. Below pH 5.5 the casein micelles begin to swell and, as almost all of the CCP is dissociated, start to precipitate. This precipitation leads to a sufficient decrease in the voluminosity of casein micelles and the formation of clusters and chains that link together to form a gel, composed of a continuous three-dimensional network with the milk serum containing whey proteins, lactose, and salts entrapped as liquid phase. Electron microscopy studies show the particulate character of acidified milk gels with empty spaces or pores in the network where the serum was entrapped (Jaros and Rohm, 2003).

Incubation of set yogurt takes place in retail containers until the required pH (around 4.4–4.7) is reached, leading to an undisturbed gel. The continuous, viscoelastic gel network consists of aggregated spherical casein particles forming a continuous structure enclosing fat globules and serum. From a structural point of view, yogurt belongs to particulate gels with disordered structures (Jaros and Rohm, 2003).

Stirred yogurt is made by inoculating and incubating milk in large fermentation vessels. The gel formed is then gently stirred to obtain a smooth and viscous but still pourable product, which is finally packed. By breaking up the gel, a highly viscous, non-Newtonian liquid is formed, which shows a strong shear rate and time-dependent flow behavior (Jaros and Rohm, 2003).

17.3 YOGURT DRINK

Apart from set yogurt and stirred yogurt, there has been an increasing demand for yogurt drinks consisting of yogurt mixed with skimmed milk, whey, or water. Drinking yogurt is produced from low-solid milk on the basis of the stirred manufacture process, or regular stirred yogurt that is diluted to some extent (Jaros and Rohm, 2003). Plain yogurt drinks are preferred in Central Asia, Anatolia, the Balkans, and the Middle East, while yogurt drinks with fruits and sweeteners are mostly consumed in Europe and the United States (Çolakoğlu and Gürsoy, 2011).

Ayran is a fermented milk product produced by the addition of water to yogurt (homemade) or by the addition of Streptococcus thermophilus and Lactobacillus delbrueckii ssp. bulgaricus to standardized milk (industrially produced) (TFC, 2009). It is a traditional yogurt drink in Turkey preferred as cold drink especially in the summer. Esfandiari et al. (2016) stated that ayran is known by different names in different countries, for instance, “doogh” in Iran and “lassi” in India. They also mentioned that doogh is made from set yogurt, whereas there is no such restriction/limitation for homemade ayran in Turkey.

The chemical composition of ayran depends on the type of milk used, the efficiency of fat removal, and the dilution rate (Kabak and Dobson, 2011). If ayran is obtained from yogurt, then it is prepared by blending yogurt with water (30%–50%) and salt (0.5%–1%) (Köksoy and Kilic, 2003).

Industrial production of ayran can be carried out by two different methods. It can be produced either by the addition of water to yogurt or the addition of water to milk first and then fermentation of diluted milk (Kocak et al., 2006; Kocak and Avsar, 2009). As a first stage, raw milk is standardized in terms of fat (1.5% for full fat, 0.8% for half-fat, 0.15% for fat free). In the first method, standardized milk is diluted with water until 8% of total solid content is obtained. In the second method, milk is homogenized and pasteurized (Kocak and Avsar, 2009). By pasteurization, it is possible to obtain ayran with good microbiological quality (Kocak and Avsar, 2009; Sen and Küplülü, 2004). Pasteurized milk is inoculated with yogurt starter cultures (S. thermophilus and L. delbrueckii ssp. bulgaricus) and incubated until a pH 4.2–4.4 is obtained. Then the fermented samples are cooled to end the fermentation.
and development of acidity (Köksoy and Kilic, 2003; Kocak and Avsar, 2009). Adequate water is added to fermented milk in the first method, until the total solid content of the fermented sample reaches 8%. Next, salt (0.5%) is added. Industrially produced ayran is bottled in glass or polypropylene or polystyrene plastic containers (Sen and Küplülü, 2004).

The composition of ayran has been reported by several authors as total dry matter (1.07%–11%), protein (1.44%–3.48%), salt (0.17%–1.75%), and fat (0.1%–3%). The titratable acidity is 0.4%–1.73% and the pH varies between 3.44 and 4.44 (Gülmez et al., 2003; Kocak et al., 2006; Sanli et al., 2011; Sen and Küplülü, 2004; Patir et al., 2006; Tamucay-Öziünlü and Kocak, 2010). Different manufacturing techniques affect acetaldehyde content but these differences are not detectable in sensory analysis (Kocak et al., 2006).

### 17.3.1 FUNCTIONAL PROPERTIES OF AYRAN

Ayran is an easily digestible and highly valued drink with high vitamin and calcium content. It appears that it is possible to develop functional properties of ayran, which already has beneficial properties. Studies investigating the functional properties of yogurt or fermented milk drinks are summarized in Table 17.1. Some studies have focused on functional properties such as probiotic characteristics.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Type</th>
<th>Added Ingredient</th>
<th>Results</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ayran</td>
<td>Laboratory-made from milk</td>
<td>Inulin or fat replacer</td>
<td>Higher aroma components compared to control</td>
<td>Kök-Taş and Güzel-Seydim (2010)</td>
</tr>
<tr>
<td>Fermented milk drink</td>
<td>Commercial product</td>
<td><em>Lactobacillus casei immunitas</em> DN-114001</td>
<td>Higher amino acid composition compared to yogurt and kefir samples</td>
<td>Irigoyen et al. (2012)</td>
</tr>
<tr>
<td>Strawberry yogurt drink</td>
<td>Laboratory-made</td>
<td>Milk fat or structured lipid emulsion</td>
<td>No significant effect on the primary measures of food intake or appetite</td>
<td>Smit et al. (2012)</td>
</tr>
<tr>
<td>Yogurt drink</td>
<td>Commercial product</td>
<td>Phytosterol esters</td>
<td>Lower meal cholesterol</td>
<td>Amiot et al. (2013)</td>
</tr>
<tr>
<td>Yogurt drink</td>
<td>Commercial product, low caloric, low fat</td>
<td>Plant sterol</td>
<td>Optimal cholesterol-lowering effect when it is in conjunction with a meal</td>
<td>Keszthelyi et al. (2013)</td>
</tr>
<tr>
<td>Yogurt drink</td>
<td>Prepared from skim yogurt, fruit juice,</td>
<td>Sucralose, acesulfame-K (noncaloric</td>
<td>SS yogurt drinks preferred over NS yogurt drinks</td>
<td>Griffioen-Roose et al. (2013)</td>
</tr>
<tr>
<td></td>
<td>calcium, aroma, and vitamin B2, B6, B12</td>
<td>sweetened, NS) or sucrose (sugar</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fermented milk</td>
<td>Laboratory-made</td>
<td>Probiotic bacteria</td>
<td>Favored over normal yogurt drink</td>
<td>Tonguç et al. (2013)</td>
</tr>
<tr>
<td>Ayran</td>
<td>Commercial product</td>
<td>Pectin (the addition is not for antioxidant activity)</td>
<td>Provides antioxidant activity</td>
<td>Najgebauer-Lejko and Sady (2015)</td>
</tr>
</tbody>
</table>
Kök-Taş and Güzel-Seydim (2010) have produced ayran by addition of inulin as a prebiotic and *L. acidophilus* and *Bifidobacterium* spp. as probiotic organisms. They have reported that the product had good taste, appearance, and higher *Lactobacillus* bacterial count. Tonguç et al. (2013) reported that probiotic microorganisms added to fermented milk were more favorable compared to normal yogurt drink in terms of acidity, aroma, and sensory properties.

In addition, Khalil (2013) added pomegranate juice to obtain a functional yogurt drink. The product consisted of carboxymethylcellulose for controlling texture. The author reported that adding pomegranate juice increased the antioxidant activity of yogurt drink, whereas it had adverse effects on rheological characteristics and serum separation.

Jacobsen (2015) stated that various food products including drinking yogurt may be enriched by n-3 polyunsaturated fatty acids. Mei et al. (2014) reported yogurt drink made from brown yogurt using fermented sweet rice.

It appears that efforts for making ayran or fermented milk drinks have focused on additions of probiotics or prebiotics. In some of these studies, the sensory properties and/or rheological properties were also measured.

### 17.3.2 Rheology of Ayran

The rheological behavior of a food matrix is an important physical property that has a direct association with product overall quality and processing characteristics, as well as on consumer acceptability (Yegin and Fernandez-Lahore, 2012). In addition, to determine any given component functionality in product development, to assess shelf-life of the food and to evaluate the food texture by correlating them to sensory properties require rheological data (Steffe, 1996). In summary, rheology is related to food acceptability, food processing, and handling (Bourne, 1982).

Studies related to the rheology of ayran-yogurt drink are given in Table 17.2. The rheological studies showed that ayran exhibits pseudoplastic (shear thinning) or thixotropic behavior depending on its fat content (Köksoy and Kilic, 2003; Lokumcu et al., 2002). In a study, whole ayran had pseudoplastic behavior whereas light ayran that does not contain fat exhibited nearly Newtonian behavior (Bayraktaroğlu and Obuz, 2008). In another study it was reported that it is unknown whether any potential differences existed in the physical, chemical, and microbiological properties of ayran samples arising from yogurt properties produced by different methods (Kocak et al., 2006). Lokumcu et al. (2002) studied the rheological properties of ayran obtained from local markets in Istanbul. They reported that *K* and *n* values of ayran samples at 9.5°C depended on their fat contents between 0.07 and 0.70 Pa s*n* and 0.38–0.66, respectively. They concluded that the rheological properties of ayran samples sold in Istanbul varied because of different compositions of products and/or manufacturing technologies.

Köksoy and Kilic (2003) prepared ayran in the laboratory by the traditional method of mixing yogurt with water and salt. They found that *K* values of ayran samples decreased from 1.214 to 0.018 Pa s*n* with the addition of salt up to 1% and water up to 50% at 10°C. In contrast, *n* values increased from 0.297 to 1.004 at 10°C with the addition of the same amount of salt and water (Köksoy and Kilic, 2003). These results showed how components affect the rheological properties of ayran. Janhoj et al. (2008) studied rheological characterization of acidified milk drinks (AMDs) at 12°C. They specified AMDs that did not contain salt as a special variety of the Turkish product ayran. They reported that *K* and *n* values of AMD acidified with lactic acid bacteria as 0.0437 (the unit of *K* was not given) and 0.6161, respectively. This sample had 2% nonfat milk solids and 0.5% pectin. The *K* and *n* values of the sample...
<table>
<thead>
<tr>
<th>Sample Name</th>
<th>Type</th>
<th>Additives for Viscosity</th>
<th>Viscosity</th>
<th>Rheological Parametersa</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ayran</td>
<td>Laboratory-made from yogurt</td>
<td>Pectin</td>
<td>7.33 s/10 cm for control 23.77 s/10 cm for the sample with 0.8% pectin</td>
<td>–</td>
<td>Atamer et al. (1999)</td>
</tr>
<tr>
<td>Ayran</td>
<td>Commercial products</td>
<td>None</td>
<td>–</td>
<td>(K = 0.07–0.70 \text{Pa s}^n, n = 0.66–0.38)</td>
<td>Lokumcu et al. (2002)</td>
</tr>
<tr>
<td>Ayran</td>
<td>Laboratory-made from yogurt</td>
<td>None</td>
<td>–</td>
<td>(K = 0.508 \text{Pa s}^n, n = 0.466) for the sample with 0.5% salt and 30% water addition</td>
<td>Köksoy and Kılıç (2003)</td>
</tr>
<tr>
<td>Ayran</td>
<td>Laboratory-made from yogurt</td>
<td>Low methoxyl pectin</td>
<td>Apparent viscosity = 15 mPa s at 55 s(^{-1}) with thixotropy of 50Pa s</td>
<td>(K = 37 \text{mPa s}^n, n = 0.77) for control sample All gum additions increased consistency indexes</td>
<td>Köksoy and Kılıç (2004)</td>
</tr>
<tr>
<td>Ayran</td>
<td>Laboratory-made from milk powder</td>
<td>None</td>
<td>1.7 mPa s</td>
<td>–</td>
<td>Koçak et al. (2006)</td>
</tr>
<tr>
<td>Ayran</td>
<td>Laboratory-made from yogurt</td>
<td>Transglutaminase for textural modification</td>
<td>15 mPa s for the control, 25 mPa s for the sample with transglutaminase</td>
<td>–</td>
<td>Şanlı et al. (2011)</td>
</tr>
<tr>
<td>Dahi (Indian yogurt) drink</td>
<td>Laboratory-made from dahi powder</td>
<td>Guar gum and locust bean gum</td>
<td>2 Pa s for the sample with 0.445 g guar gum + 0.905 g locust bean gum + 20 g dahi powder + 200 g water</td>
<td>–</td>
<td>Routray et al. (2011)</td>
</tr>
<tr>
<td>Ayran</td>
<td>Laboratory-made from milk</td>
<td>None Sample from thermosonicated milk</td>
<td>174 mPa s at 100 rpm 187–244 mPa s at 100 rpm</td>
<td>(K = 7.11 \text{mPa s}^n, n = 0.568)  (K = 5.85–6.74 \text{mPa s}^n, n = 0.672–0.808)</td>
<td>Gürsoy et al. (2016)</td>
</tr>
<tr>
<td>Yogurt drink</td>
<td>–</td>
<td>Agar</td>
<td>–</td>
<td>Significant increase (G') and (G'')</td>
<td>Tian et al. (2016)</td>
</tr>
</tbody>
</table>

\(^a\text{K, consistency index (Pa s}\(^n\)); \(n\, \text{flow behavior index (–)}; \(G'\, \text{elastic modulus (Pa)}; \(G''\, \text{viscous modulus (Pa)}.\)
with 2% nonfat milk solids and 0.5% carboxymethylcellulose were 0.0280 (the unit of $K$ was not given) and 0.8185, respectively.

The structural characteristics of yogurt drinks are viscosity, gel structure, water-holding capacity, particle size, particle density, and particle distribution. These affect the characteristics for consumer preferences. Serum separation and consistency have been monitored for commercial yogurt drink manufacturing (Gürsoy et al., 2016).

The main textural defects are low viscosity and serum separation (up to 30%). Serum separation occurs in fermented milk products because of the aggregation of casein micelles to particles and sedimentation of casein particles during storage. The presence of salt in ayran may cause more serum separation compared to other fermented milk beverages. The addition of increasing levels of salt and water was found to increase the serum separation in ayran (Köksoy and Kilic, 2003). Serum separation can be prevented by the addition of hydrocolloid stabilizers such as pectin, guar gum, and gelatin (Köksoy and Kilic, 2004), or addition of transglutaminase (Sanli et al., 2011). The significant effect of stabilizers on taste, odor, consistency, and overall acceptability was determined (Köksoy and Kilic, 2004). Transglutaminase has been used to modify functional properties of food proteins. The addition of transglutaminase at a level of 1 TGase/g had no significant effect on the flavor or chemical properties of ayran (Sanli et al., 2011).

Tamucay-Özünlü and Kocak (2010) investigated the effects of different heat treatments of milk (at 75, 85, and 95°C for 5 min) on various properties of ayran. The acetaldehyde content, serum separation, and viscosity were significantly affected. Heat treatment at 95°C was recommended to obtain the highest viscosity and the least serum separation.

Bayraktaroglu and Obuz (2008) stated that additions of guar gum, locust bean gum, and whey powder to nonfat ayran samples gave better quality characteristics in terms of texture and consistency. Yu (2015) reported that addition of pectin, carboxymethylcellulose, xanthan, and guar gum provided stable and palatable yogurt drinks. Godarzi et al. (2015) studied the optimization of formulation fermented drink yogurt with fruit juice, fruit juice concentrates, and stabilizers. Kucukcetin et al. (2012) studied the effect of casein to whey protein ratio of skim milk on the physical properties of ayran. They reported that when the casein:whey ratio increased, the number of grains, the mean grain perimeter, and visual roughness decreased; however, serum separation and viscosity increased.

Rheological studies on ayran and other yogurt and fermented milk drinks have mostly focused on inhibiting serum separation and increasing viscosity. To do this, the addition of thickeners with a stabilizing effect is the most common method including performing sensory analysis. The most accepted or preferred product in terms of structure, mouthfeel, aroma, and flavor has not been correlated with its functionality using the understanding of structural mechanisms of the system.

17.4 CONCLUSIONS

Textural problems in ayran such as serum separation/solid particle sedimentation, acidity development, and low viscosity affect consumer acceptance in terms of mouthfeel and appearance, and are related to quality characteristics during manufacturing, transportation, and storage. It appears that most quality problems are rheology related, which are affected by initial raw materials and composition of product, fermentation conditions, type of microorganisms, and their numbers and ratios (Altay et al., 2013). The functional properties of ayran and other yogurt drinks or fermented milk drinks appear to have an
important role as a “functional” drink. The functional properties of these drinks are affected by composition, added ingredients to the formula, and possibly manufacturing methods. It may be a good idea to investigate the functionality of ayran or other yogurt/fermented milk drinks as they are and then further analyze the effects of functional ingredients. To do so, structural mechanisms at the molecular level must be understood for rheological properties during manufacturing, storing, consuming, and functional properties in the body. After structure–function relations are well defined, then yogurt and fermented milk drinks with more functional/nontextural defects may be produced.

Apparently, more studies are needed to investigate the functional properties and added ingredients to improve functional properties, rheology and their relations for improving quality and safety of yogurt and fermented milk drinks.

REFERENCES
Çolakoğlu, H., Gürsoy, O., 2011. Effect of lactic acid adjunct cultures on conjugated linoleic acid (CLA) concentration of yoghurt drink. J. Food Agric. Environ. 9, 60–64.


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18.1 TRADITIONAL BULGARIAN DAIRY PRODUCTS

The consumption of fermented foods has increased greatly since the 1970s. This includes not only common foods such as dairy products (yogurt, cheese, buttermilk, and cream), fermented sausages, sourdoughs, fermented alcoholic beverages, vegetables, fruits, and sauces but also ethnic foods such as kefir and koumiss. Lactic acid bacteria (LAB) are among the first organisms to be used in the manufacture of these appetizing and well-accepted foods by consumers (Holzapfel, 2002). Today LAB play a crucial role in the production of fermented foods, vegetables, and meat, as well as drugs against intracellular targets.

One of the reasons for the increase in consumption of fermented dairy products is customer’s consideration that these foods are healthy and natural. The presence of billions of live cells of desirable microorganisms and their metabolic products in fermented foods does not cause any panic or distress among safety-concerned consumers. These foods have been used for thousands of years and therefore have withstood the test of time. For this reason, an important task for microbiologists is to investigate fermentation processes and microbiota responsible for their production and preserve them for further application in functional foods.

18.1.1 BULGARIA—THE MOTHERLAND OF THE FIRST WIDELY ACCEPTED PROBIOTIC FOOD

Bulgaria is a country with long traditions in production of fermented milk foods (Baltadjieva, 1993). In Europe the main purpose of food fermentations focused on food preservation by means of acid fermentation. As a result, several fermented dairy products are widely consumed. According to specific (national, regional, or artisanal) recipes, milk is fermented by either the indigenous microflora and/or added starter cultures to improve shelf-life, nutritional value, health benefits, flavor, and texture.

The food culture of countries on the Balkan Peninsula depends mainly on historic, geographical, climatic, social, and religious elements. Hence, in many parts of the Balkans, a high similarity in consumed foods can be found, especially in the group of traditional fermented products. Among these products, yogurt and cheeses are the most popular dairy foods and are consumed every day. At the same time, some products are considered as unique. The most popular product in Bulgaria is yogurt. The regional cheese market is dominated (>50%) by “white-brined cheeses,” e.g., “sirene,” “feta,” “ripened
and stored under brine until the time of consumption, followed by yellow cheese (kashkaval), butter, cream, and curd. In Bulgaria, fermented dairy products are prepared from ewe’s, cow’s, goat’s, and buffalo’s milk and/or a mix of two types of milk (usually sheep’s and cow’s milk). Recently, industrial production of some artisanal products such as “katak” has started.

According to the National Scientific Institution data, an increase in average consumption per person of households in 2013 was reported for almost all major Bulgarian dairy products, except yogurt. The most significant growth was observed in the consumption of butter (12.5%), followed by yellow cheese (5.7%) and other dairy products and sweeteners (21%). Similar to the other Balkan cultures, the per capita consumption of dairy products among Bulgarians is traditionally higher than the rest of Europe.

18.1.2 OTHER FERMENTED DAIRY PRODUCTS, ACCORDING TO THE OLD BALKANS TRADITIONS

Fermentation processes, initiated without the use of a starter inoculum, have been applied to Balkan food preservation for millennia. Their conditions are set so that the desirable microorganisms grow preferentially and produce metabolic by-products, which give the unique characteristics of the product. However, spontaneous food fermentations are neither predictable nor controllable and they are highly influenced by environmental factors (Desfossés-Foucault et al., 2013). At the same time, such types of processes have great potential for diversification of flavor, taste, and properties of the products. Good examples are “katak” (Fig. 18.1A), green cheese (Fig. 18.1B), “brano mLiako,” a special kind of yogurt (Fig. 18.1C) (Gruev, 1966), and cheese in ceramic pots (Fig. 18.1D), produced in Bulgaria (Gruev and Minkov, 1994). These products represent the national heritage and are a result of centuries-long accumulated empirical knowledge passed down from generation to generation. Some of these foods are no longer considered by consumers only in terms of taste and immediate nutritional needs but also in terms of their ability to provide specific benefits above their basic nutritional value. Thus they correspond well to the new demands for functional foods.

18.1.2.1 Katak or Krutmatch—An Old Tradition From Ancient Times

The word “katak” comes from the ancient terminology that denotes a product that facilitates the digestion of bread (Kojev, 2012). A more modern interpretation of this word means “additive,” which suggests that this product can be taken with bread or dry food (Fig. 18.1A). Thus it is a fermented milk product, with specific aroma, taste, and texture, which correlates with yogurt (especially the variant produced in southeastern Bulgaria), or to the kind of soft or cream cheeses (the katak, prepared in northern parts of Bulgaria).

18.2 “KATAK” OR “KRUTMATCH”—AN ARTISANAL DAIRY PRODUCT FROM BULGARIA, WITH IMPACT ON THE MARKET OF FUNCTIONAL FOODS

18.2.1 WHAT IS KATAK?

Katak is the most popular name of one fermented curd/yogurt-like product with a specific salted milk–acid taste, manufactured in Bulgaria for centuries (Kojev, 2012). In some regions, katak is named ahtchak (or svedene), and krutmatch (or kurtmatch) in other parts of Bulgaria—especially in the northern parts of Stara planina mountain (Fig. 18.2). All these products are a part of a big assortment of fermented foods,
which are traditionally consumed in the Balkans. Katak’s recipe is inherited from the proto-Bulgarians and has been changed over the years. Despite its different ways of preparation, this artisanal product is delicious and possesses an acid taste and specific fermented milk flavor. According to tradition, katak was primarily made from sheep’s milk in the late summer, just before the end of the lactation period (Gruev and Minkov, 1994). Nowadays, katak is prepared only in mountainous regions with developed sheep breeding (Fig. 18.2). Such a type of milk is hard to curdle and for this reason it is unprofitable for the production of traditional, Balkan white-brined and yellow cheese. Therefore mountainous farmers began to produce this unique fermented dairy product, which is distinguished from yogurt by its taste and much longer shelf-life. Different recipes for “katak” preparation can be found because of the customs of various mountainous regions, which has also led to differences in the name of this unique product.

The names “katak,” “krutmatch,” and “ahtchak” are perceived by consumers as synonymous. However, a fundamental difference in their preparation is observed; ahtchak represents a condensed and salted ewe’s milk whose fermentation is carried out by using a natural or fermentation starter or another additive according to the taste of the producer (Fig. 18.3). In katak’s (or krutmatch’s) preparation, crushed, ripened white cheese is added, whose autochthonous microflora causes secondary fermentation with beneficial effects on the ripening of the obtained mixed product (Fig. 18.3A and B).
The higher content of dry substances in milk (such as proteins and fats) leads to better texture of the product—an important requirement for both products. For this reason, late ewe’s milk is used to improve the product’s quality. If sour milk is used, it should be thickened by boiling on a slow and low heat. The usage of skimmed milk (8%–12% w/v) will save the prolonged boiling time, which may lead to unpleasant effects on organoleptic properties.

Apart from the different methods of preparation, its organoleptic properties, and the type of ripening, katak represents a peculiar transition between yogurt-like product and soft rennet cheese. Another important difference is the usage of salt in the food processing. Thus, the salted taste makes katak and ahtchak similar to cheese. Sodium chloride is not only a flavor additive, but also an active ingredient in the degradation process of LAB (cell autolysis) involved in the ripening processes. NaCl is also associated with LAB proteins/molecules from autolysis, actively influencing the biochemical processes in katak.

Katak has great potential as an appetizing and noncaloric milk product, with some characteristics of functional food (Table 18.1). Presented comparative data show that this is a well-balanced product (according to protein and fat content) like yogurt, and also possesses the taste of cheese. Moreover, katak may be a source of biologically active molecules, produced during the long fermentation processing, by lactic acid microflora. Consumers understand that the amount and combination of nutrients in consumed foods can greatly affect their health and reduce the risk of diseases.
FIGURE 18.3A
Traditional recipes for the preparation of katak in different rural regions of Bulgaria (according to Gruev and Minkov, 1994). Blue and green flashes (light gray and black in print versions)—recipes for krutmatch, prepared in the northern and northwestern parts of Bulgaria; red flashes (dark gray in print versions)—a recipe for ahtchak, prepared in the southern part of Bulgaria—the Rhodopes.
FIGURE 18.3B

Katak’s preparation according to traditional recipes, conserved and applied today in small farms in Bulgaria. 

Blue flashes (black in print versions)—recipes for krutmatch, prepared in the northern and northwestern parts of Bulgaria (a small farm in Lukovit—northern part of Bulgaria, Stara planina mountain); red flashes (gray in print versions)—a recipe for ahtchak, prepared in southern parts of Bulgaria—the Rhodope mountains—a small farm near Chirpan in the southeastern part of Bulgaria.

Data collected from B. Nikolova (UCTM, Sofia) and At. Kiskinov—Bulgarian Association of Sheep Breeding, Bulgaria.
Because of the low level of carbohydrates, especially lactose, this product can be consumed by lactose-intolerant individuals as well. Production of lactic acid during milk processing is an important means of preventing/limiting milk spoilage caused by the growth of contaminating bacteria and their enzyme activity. The acidity of katak correlates with that of industrial yogurt (pH $\sim$4.3–4.5); however, its shelf-life is much longer, up to 12 months, at low temperature (10–12°C).

Human desire for a long and healthy life needs an alternative to the present food practices of affluent modern societies. Nutritional habits should be reconsidered and (re)directed toward consumption of healthier foods, free of chemical preservatives, with smaller allergenic activities, and containing active compounds and/or living bacteria with beneficial effects on consumers. Scientists have to respond to these demands by creating new functional and dietary foods or therapeutic formulas with stable market potential. In this field, probably the most promising sources are traditional and not well-studied fermented milk products. With this aim the major part of our work was directed to the characterization of the microbiota of traditional katak (data presented in point 4 in the Section 18.2.2.1).

### 18.2.2 HOW IS KATAK PREPARED IN DIFFERENT RURAL REGIONS: FROM ARTISANAL RECIPES TO THE TECHNOLOGY OF MODERN DAIRY PRODUCTION?

Nowadays, long-lasting “katak” is made from leavened ewe’s milk. Collected data from rural regions with conserved traditions in small villages in the Rhodope and Pirin mountains in southern Bulgaria, and the mountainous and foothill regions of Stara planina mountain in northern Bulgaria (Fig. 18.2), showed two different traditional recipes for this product.
18.2.2.1 Katak or Ahtchak
According to the collected data by Prof. P. Gruev and Prof. T. Minkov (1994) the preparation of “ahtchak” (one kind of katak) is carried out using the following steps:

1. Milk is filtered through a layered cotton canvas;
2. Milk is boiled gradually on a very low heat, constantly stirred to intensify the evaporation and prevent the milk from burning;
3. Milk is heated to 95°C, and this temperature is maintained until it reaches the desired density. If late ewe’s milk is used, this is achieved when the total milk volume is reduced by 5%–10%. In modern conditions, it can be replaced by ordinary milk, but longer boiling and the addition of 5%–6% (w/v) skimmed milk are needed;
4. The boiled milk is cooled down to 80°C and stirred periodically. Homemade yogurt starters (0.5% v/v including Lactobacillus delbrueckii ssp. bulgaricus and Streptococcus thermophilus) and 2.5% (w/v) salt (NaCl) are often added to the milk. However, some villages prepare katak without any starter addition. Only salt and environmental lactic acid microflora during the first step of slow fermentation are responsible for the formation of specific organoleptic properties (Fig. 18.3). The salted yogurt taste of milk in the beginning is shifted to the sensory properties of the soft cheeses during the ripening process. In some regions, starters for white-brined cheese are used, which differ in their content to those for yogurt. It consists of Lactobacillus casei and Lactococcus lactis. The two starter cultures are cultured at different temperatures, 37–40°C (for yogurt starter) and 30°C (for cheese starter). When keeping these optimal temperatures, the product passes through a specific stage typical for cheese making, i.e., formation of typical structure, based on small milk grains;
5. The boiled and salted milk is poured into suitable containers (special jars or pots) and undergoes prefermentation at 15–18°C for 4–5 days. Then it is matured at a temperature below 10°C (in a cellar or a refrigerator) for 30–40 days;
6. The product is placed in a special ceramic jar/closed glass jar (without any additional treatment) and can be stored until the beginning of spring (next lactation period); it possesses excellent taste, which often defines it as a cheese in a jar (Fig. 18.3B).

Collected additional data showed that nowadays recipes of katak are well conserved and are applied with some modifications in small farms (Fig. 18.3B). The addition of cheese to the long and slowly boiled milk is preserved as a step in the preparation; however, the conditions of slow fermentation and the ripening period at a low temperature may vary in different families, farms, and regions (Fig. 18.3B). Therefore a comparative study of different artisanal samples of katak will be highly informative.

18.2.2.2 Katak or Krutmatch (Kurtmatch)
The principal difference between “ahtchak” and “katak” is that crushed ripened cheese is usually added to boiled milk in 50:50 (v/v) or other proportions of milk to cheese (Gruev and Minkov, 1994). An important condition is that added cheese is in a fine-grained form because of the preserved small lumps/grains of well-ripened cheese.

Presently, there is no production of authentic katak in Bulgaria. Industrial recipes combine some elements from the old traditional recipe, but the taste is quite different and the shelf-life of the product is only 21 days (Baltadjieva, 1993). The original homemade katak, prepared according to the
technology of krutmatch with salt before the fermentation (Fig. 18.4), keeps its quality and taste up to 6 months at 10–15°C.

Naturally occurring yeasts and LAB (nonstarters) during the preparation of katak play a major role in the formation of the final product. Thus a variety of tastes and geographically dependent specificities of the production technology can be found. However, very limited data exist on the microflora
responsible for the formation of the typical cheese-like milk–acid taste of this Balkans product. With this aim, several samples of traditionally produced homemade katak were collected from rural regions of Bulgaria and their lactic acid microflora was examined.

18.3 Microbiota of Katak

18.3.1 Initial Characterization of LAB Microbiota in Artisanal Samples of Katak from Different Regions of Bulgaria

There is no available information on katak’s starter/nonstarter microbiota. In the last 6 years, the Laboratory of Probiotics and Lactic Acid Bacteria (The Stephan Angeloff Institute of Microbiology, BAS, Bulgaria) has started a new research project on katak’s microbiota. The main objectives are isolation of LAB from homemade samples of katak, identification of newly isolated strains, and initial characterization of functional activities and probiotic potential of autochthonous LAB microbiota.

Katak samples were collected from small farms in the Rhodope mountains (mountain areas above the towns of Trigrad, Ardino, Kardzhali, and Zlatograd) and central Stara planina (near the regions of Lukovit, Gabrovo, Sevlievo, Apriltsi, Lovech, and Yablanitsa) (Fig. 18.2). Seasonal production (only in late summer) is the reason for including a limited number of samples and to keep a freeze-dried homemade original katak from the region of Gabrovo. Different selective media, de Man, Rogosa and Sharpe (MRS, Merck, Germany), Rogosa (Himedia, India), M17 (Merck, Germany), L-S differentiation agars (Scharlau, Spain), and a previously described protocol (Danova et al., 2012), were used to obtain pure cultures of LAB.

The complexity and diversity of katak’s microbiota are still not characterized. Thus utilization of different selective media was necessary to isolate and characterize the lactic acid microflora of this traditional dairy product. Our results showed a notable viability and diversity of bacteria and yeasts in collected katak samples isolated from different ecological rural regions. The quantitative microbiological analyses of all samples of katak (until the expiry date and one freeze-dried sample) showed good viability, in the range of $1.3 \times 10^6$–$4 \times 10^7$ cfu/g. Especially high microbial variety was observed in the case of submerged inoculation of serial dilutions from katak in Petri dishes with MRS and Rogosa agar, cultivated under anaerobic conditions (GasPak 100, Anaerobic System, BD Bioscience, USA) at 37°C for 48–72 h.

As a result of the isolation procedure, a collection of 60 randomly selected pure cultures was obtained. They were Gram (+), catalase and oxidase negative, nonsporulating, nonmotile microorganisms, which corresponded to the characteristics of LAB (Kandler and Weiss, 1986a,b). The existence of a morphological variety of cocoidal and rod-shaped forms can also be observed. All isolates grew well at 30–37°C in anaerobic conditions and can be phenotypically differentiated as belonging to the genera *Lactobacillus*, *Streptococcus*, and *Pediococcus*. Twenty-five were identified as *Lactobacillus* spp. according to classical cultural, physiological, and biochemical characteristics. Kirilov et al. (2011) reported that lactobacilli, lactococci, and streptococci represent half of 107 newly isolated strains from cheeses and katak samples from the Thracian valley and mountain region of Stara planina mountain (western part of Bulgaria) and from the mountain region of the Rhodope mountains (southern Bulgaria). However, enterococci have been estimated as dominant among LAB isolates. Both phenotypic and genetic methods were applied for the *Enterococcus* identification to genus and species levels (Kirilov et al., 2011).
Tserovska et al. (2002) isolated two strains from katak. On the basis of their morphological, cultural, and physiological–biochemical characteristics, they were identified as *Pediococcus acidilactici* and *Pediococcus pentosaceus* (Tserovska et al., 2002). The strains were deposited in the National Bank for Industrial Microorganisms and Cell-Cultures (NBIMCC) with accession numbers 3437 and 3545, respectively.

Some samples of katak-type krutmatch prepared according to the recipe presented in Fig. 18.3 (especially in the early stages of the fermentation and ripening processes) showed a significant presence of yeasts, which contributed to gas and specific aroma formation.

Eight *Lactobacillus* strains were preselected from the collection for additional characterization. Four of them, the strains KR3, KR4, KR51, and KR53, originated from homemade samples of katak–ahtchak (Fig. 18.2) from the Rhodope mountains (the region near Trigrad). They possess a strong antifungal activity, estimated in vitro in one of our screening studies (Tropcheva et al., 2010). The other strains, named S1, S2, S3, and S4, were isolated from samples of katak–krutmatch (Fig 18.3B) prepared in Stara planina mountain (the region near Sevlievo) (Fig. 18.2).

The identification system API LAB 50CHL (Biomerieux, France) was used to determine the initial species affiliation of the selected strains (Tropcheva et al., 2009). According to their biochemical profiles they belong to the species *Lactobacillus brevis* and *Lactobacillus plantarum*, with varying degrees of reliability, and the species affiliation was determined by molecular methods (Table 18.2).

Seven out of the 25 isolates were identified to the species level, according to the modern polyphasic approach, combining physiological and API LAB 50 CH biochemical tests with different molecular methods [species-specific polymerase chain reaction (PCR), random amplified polymorphic DNA-PCR, and 16S rDNA sequence analyses]. To our knowledge, these are the first data on molecular characterization of *Lactobacillus* microbiota in “katak.” The full compliance of phenotypic characteristics and results from the molecular genetic tests justifies the identification of strains KR3, KR4, KR51, and KR53 as *L. brevis* (Tropcheva et al., 2014). Despite the application of the gold standard in bacterial taxonomy (16S rDNA sequencing), some strains are identified with a low level of similarity (Table 18.2). Additional molecular–genetic characterization, however, is needed and is still in progress. Further metagenomic analysis will give more reliable information about katak’s microbiota.

Despite the low number of identified *Lactobacillus* from the group of isolates, a predominance of heterofermentative *Lactobacillus* species (*L. brevis*, *Lactobacillus fermentum*, *Lactobacillus casei* group—*Lactobacillus rhamnosus*) could be confirmed.

The species *L. brevis* is reported in other traditionally fermented foods by API LAB minikits (Ali and Mustafa, 2009) and by other taxonomic approaches. For example, Skelin et al. (2012), using species-specific primers, proved the existence of *L. brevis* in cheeses from Istria. *L. brevis* is also a participant in the microflora of Cheddar cheese (Desfossés-Foucault et al., 2013). It is a widespread representative of obligate heterofermentative lactobacilli, which can also be isolated from wine and beer (Fujii et al., 2005), sauerkraut, pickles, the traditional Korean product kimchi (United Nations FAO, 2007), and ray sourdough (Dobreva-Josifova, 2012). However, there are no data for the presence of *L. brevis* in yogurt or yogurt-like products. Therefore its presence in Bulgarian yogurt-like product “katak” was unexpected.

*L. brevis* is found to be a starter culture of kefir’s grains “tibicos,” responsible for the synthesis of the structurally important component dextran (Pidoux, 1989) and a nonstarter culture of dairy products made from raw milk (Kagli et al., 2007).
A combination of classical phenotypic and molecular methods (multiplex PCR, according to Torriani et al., 2001, species-specific PCR, and 16S rDNA sequencing) revealed the presence of *L. plantarum* and *L. fermentum* species (Table 18.2).

*Lactobacillus plantarum* is one of the widespread LAB species, presented as autochthonous microbiota in several fermented foods. It is also reported as part of the nonstarter microflora in the stage of maturation of different types of cheeses, influencing effectively the sensory properties of the products, even after their production (Corsetti and Gobbetti, 2002). The highest number of *L. plantarum* was reported in Italian (Pecorino), Spanish (Manchego, Cabrales, and Roncal), Portuguese (Picante), Irish (Cheddar), and Flemish (Gauda) cheeses (Corsetti and Gobbetti, 2002; Poveda et al., 2003; Ouadghiri et al., 2005; Abriouel et al., 2008; Van Hoorde et al., 2008). However, as a dominant species it is discovered mainly in Greek cheeses feta and Teleme (Tzanetakis and Litopoulou-Tzanetaki, 1992; Xanthopoulos et al., 2000; Rantsiou et al., 2008).

The species from the *L. plantarum* group (*L. plantarum*, *Lactobacillus pentosus*, and *Lactobacillus paraplantarum*) have been reported in Pecorino Crotonese cheese (Randazzo et al., 2009), Lighvan (Ghotbi et al., 2006), and Danbo cheese (Antonsson et al., 2003). Skelin et al. (2012) reported *L. plantarum* as a dominant species in cheeses from Istria. De Angelis et al. (2001) studied 12 Italian cheeses and found that 32% of the nonstarter microflora is represented by *L. plantarum*. More data are added to the presence of this species in a variety of cheeses such as Pecorino Romano (Dellaglio et al., 1981), Cheddar (Thomas, 1987), Belgian soft cheese (Poffe and Vanheusden, 1986), Stilton and Gouda (Van Hoorde et al., 2008), Kopanisti—traditional Greek cheese (Tzanetakis et al., 1987), feta (Tzanetakis and Litopoulou-Tzanetaki, 1992; Rantsiou et al., 2008), sheep cheese (Oneca et al., 2003), cheese Alberquilla (Abriouel et al., 2008), Manchego cheese (Poveda et al., 2003), and Moroccan soft, white cheese (Ouadghiri et al., 2005). Therefore the presence of this species in katak was expected. Moreover, our recent study (Danova, 2015) using direct multiplex PCR (for the *L. plantarum* group, according to Torriani et al., 2001) with target total DNA extracted from homemade yogurts and katak obtained positive indication for *L. plantarum* being present in the examined dairy samples (Fig. 18.5). The PCR assay included one sample of yogurt from Rila mountain (southwest part of Bulgaria), 12 samples of artisanal white-brined cheeses,

### Table 18.2 Identification of *Lactobacillus* Strains From Traditional Bulgarian “Katak”

<table>
<thead>
<tr>
<th>Strain</th>
<th>Species Affiliation</th>
<th>Sequence (%) Similarity</th>
<th>Accession Number in NCBI</th>
</tr>
</thead>
<tbody>
<tr>
<td>KR3</td>
<td><em>Lactobacillus brevis</em></td>
<td>100</td>
<td>HM568883.1</td>
</tr>
<tr>
<td>KR4</td>
<td><em>L. brevis</em></td>
<td>100</td>
<td>HM568884.1</td>
</tr>
<tr>
<td>KR51</td>
<td><em>L. brevis</em></td>
<td>100</td>
<td>HM568885.1</td>
</tr>
<tr>
<td>KR53</td>
<td><em>L. brevis</em></td>
<td>100</td>
<td>HM568886.1</td>
</tr>
<tr>
<td>S1</td>
<td><em>Lactobacillus spp.</em></td>
<td>No similarity</td>
<td>The submission in NCBI-GenBank is still in progress</td>
</tr>
<tr>
<td>S2</td>
<td><em>Lactobacillus spp.</em></td>
<td>91</td>
<td></td>
</tr>
<tr>
<td>S3</td>
<td><em>Lactobacillus plantarum</em></td>
<td>94</td>
<td></td>
</tr>
<tr>
<td>S4</td>
<td><em>Lactobacillus fermentum</em></td>
<td>96</td>
<td></td>
</tr>
</tbody>
</table>

*16S rDNA sequencing of new isolates and percentage of sequence similarity after BLAST analysis with the available data on NCBI-GenBank.*
**18.4 ANTIMICROBIAL ACTIVITY OF *LACTOBACILLUS* FROM BULGARIAN KATAK**

Determination of the spectrum of antimicrobial activity is an essential step in the characterization of LAB and their probiotic potential. Production of promising antimicrobial active metabolites has been reported for various LAB of different origins (Amant et al., 2002; Tannock, 2003). It was found that *Lactobacillus* spp. possess a broad spectrum of antagonistic activity against various Gram (+) and Gram (−) pathogens (Georgieva et al., 2008; Volzing et al., 2013), food spoilage bacteria (Tropcheva et al., 2014), and/or other not so closely related microorganisms (Elegado et al., 2004; Suma et al., 1998; Todorov and Dicks, 2005).

---

**FIGURE 18.5**

Multiplex polymerase chain reaction (PCR) analysis (according to Torriani et al., 2001) for the presence of the species *Lactobacillus plantarum* in homemade samples from cheese, katak, and yogurt. M, Molecular marker—50 bp (Fermentas); 1, *L. plantarum* ATCC 14917T; 2, *Lactobacillus pentosus* ATCC 8041; 3, *Lactobacillus paraplantarum* ATCC 700211T; 4, sheep cheese; 5, sheep cheese; 6, cheese from a mixture of cow’s milk; 7, goat cheese; 8, sheep cheese; 9, sheep cheese; 10, sheep cheese; 11, cheese from a mixture of cow’s and sheep’s milk; 12, cow’s milk cheese; 13, cow’s milk cheese; 14, buffalo cheese; 15, sheep cheese; 16, katak, made from Trigrad (the Rhodopes); 17, sheep yogurt.

made according to traditional technology without starters added, and one sample of katak (the same that was the source for isolation of the strains KR3, KR4, KR51, and KR53). This sample was made according to the traditional recipe for ahtchak (Fig. 18.3B).

The source of *L. plantarum* could be the raw milk, which is often preferred in the traditional preparation of homemade cheese. A study of the microflora of raw milk for the production of Bulgarian white-brined cheese detected *L. plantarum* strains (Chomakov and Kirov, 1973). The addition of cheese to initiate katak’s fermentation (Fig. 18.3A and B) is probably responsible for the presence of *L. plantarum* among its microflora. However, this strain is absent in industrially prepared fermented products (yogurt, cheese, and katak) whose main purpose is the standardization and preservation of product quality (Baltadjieva, 1993).

Bulgaria has not conducted any systematic research on nonstarter LAB of widely consumed dairy products, such as yogurt, white-brined cheese, and katak. The new information about species’ variety has important scientific and practical significance. The obtained data allowed the additional characterization of LAB microflora of this appetizing but not well-studied Bulgarian milk product. In this respect it is also very important to estimate a number of technologically relevant properties and biological activities of katak’s microflora.
The LAB microflora of katak has never been tested for antimicrobial activity. At the same time it is a fermented product with a much longer shelf-life than yogurt (up to 2–4 months for ahtchak and 12 months for krutmatch at 4–10°C). The combination of specific technology (Fig. 18.3) and biological activity of autochthonous microflora, present in the fermentation and ripening process, is an important factor that contributes to long shelf-life and stable organoleptic properties of katak. To estimate this hypothesis, several in vitro tests for the determination of the antimicrobial spectrum of katak’s microbiota were carried out.

18.4.1 ANTIBACTERIAL ACTIVITY OF \textit{Lactobacillus} ISOLATED FROM BULGARIAN KATAK

An initial step in characterization of antagonistic activity of strains from katak is the in vitro test against different test bacteria. The screening of all 25 isolates was made in parallel against Gram (+) \textit{Bacillus subtilis} ATCC 6633 and Gram (−) bacteria \textit{Escherichia coli} HB 101 (IMSA- BAS collection). The strains that showed good antimicrobial activity against both test strains were further subjected to another agar-diffusion assay: (1) agar-well diffusion method (Table 18.3A) and (2) direct agar-spot method (Table 18.3B). The enteropathogen test cultures (in exponential growth phase) were used in the tests (Table 18.3B).

This antagonistic activity is mediated directly by the production of inhibitory substances such as lactic acid, hydrogen peroxide, bacteriocins, and other unidentified compounds. Moreover, bacteriocinogenic LAB may compete with pathogens for the binding sites on the epithelial cell surface and establish an effective barrier against foodborne pathogens. Candidate probiotic strains with a broad spectrum of activity have great potential to prevent infections caused by enteropathogens (Simmering and Blaut, 2001).

18.4.2 \textsc{In vitro tests for estimation of antifungal activity of lab from katak}

Seven fungal species, \textit{Aspergillus flavus} (NBIMCC), \textit{Aspergillus awamori} K1 (DB-SU), \textit{Aspergillus niger} A3 (DB-SU), \textit{Trichoderma viride} (DB-SU), \textit{Trichoderma harzianum} (DB-SU), \textit{Penicillium}

<table>
<thead>
<tr>
<th>Table 18.3A</th>
<th>In Vitro Antibacterial Activity of Lactobacilli Isolated From Bulgarian Katak (Krutmatch)</th>
</tr>
</thead>
</table>
| Strain      | \begin{tabular}{l|c|c|c|c|c|c}
|             | \multicolumn{3}{c|}{Antibacterial Activity (in mm Sterile Zone) Against:} & \multicolumn{3}{c}{Bacillus subtilis ATCC 6633} \\
|             | \textit{Escherichia coli HB 101} & \textit{Bacillus subtilis ATCC 6633} & \textit{Bacillus subtilis ATCC 6633} & \textit{Bacillus subtilis ATCC 6633} & \textit{Bacillus subtilis ATCC 6633} & \textit{Bacillus subtilis ATCC 6633} \\
|             | aCFS | nCFS | fM | aCFS | nCFS | fM \\
| S1          | 12   | 0    | 14 | 23   | 0    | 14 \\
| S2          | 10   | 0    | 13.5 | 17.5 | 0    | 14 \\
| S3          | 12   | 0    | 14$^a$ | 13.5 | 0    | 10$^a$ \\
| S4          | 11   | 0    | 13.5 | 14.25 | 11$^a$ | 11 \\
| \multicolumn{7}{l}{$^a$Bacteriostatic effect on the test cultures; aCFS, acid cell-free supernatants from exponential cultures in MRS broth; nCFS, neutralized cell-free supernatants with 5M NaOH to pH ~6.0–6.5 (to eliminate the inhibitory effect of the produced lactic acid); fM, fermented milk.} |

$^a$Bacteriostatic effect on the test cultures; aCFS, acid cell-free supernatants from exponential cultures in MRS broth; nCFS, neutralized cell-free supernatants with 5M NaOH to pH ~6.0–6.5 (to eliminate the inhibitory effect of the produced lactic acid); fM, fermented milk.
Table 18.3B  In Vitro Tests for Antibacterial Activity of Lactobacilli Isolated From Bulgarian Katak (Ahtchak)

<table>
<thead>
<tr>
<th>Strain</th>
<th>Escherichia coli ATCC 11775</th>
<th>Enterobacter aerogenes ATCC 13048</th>
<th>Salmonella enteritidis ATCC 13076</th>
<th>Staphylococcus aureus ATCC 12600</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>aCFS</td>
<td>nCFS</td>
<td>fM</td>
<td>aCFS</td>
</tr>
<tr>
<td>KR3</td>
<td>8(^a)</td>
<td>0</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td>KR4</td>
<td>10(^a)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>KR51</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>9</td>
</tr>
<tr>
<td>KR53</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>5(^a)</td>
</tr>
</tbody>
</table>

\(^a\)Bacteriostatic effect on the test cultures; aCFS, acid cell-free supernatants from exponential cultures in MRS broth; nCFS, neutralized cell-free supernatants with 5M NaOH to pH ~6.0–6.5 (to eliminate the inhibitory effect of the produced lactic acid); fM, fermented milk.
claviforme (IMB-BAS), and Fusarium graminearum (NBIMCC), were used. The dual agar-plate method (Hassan and Bullerman, 2008) was used in laboratory tests (in triplicate) as a good model system that allows the estimation of the capacity of a growing LAB culture to inhibit the growth of different molds. All tested 24-h exponential cultures showed a strain-specific spectrum of antifungal activity (Figs. 18.6 and 18.7). The L. brevis KR3, KR4, KR51, and KR53 strains demonstrated a broad spectrum of activity (Tropcheva et al., 2010, 2014).

18.4.3 DUAL LAYER AND MONOLAYER AGAR PLATE ASSAYS WITH LACTOBACILLUS FROM SAMPLES OF TRADITIONAL BULGARIAN KATAK

A modification of the method was applied to carry out the initial screening of antifungal activity. Each exponentially growing LAB culture was solidified in MRS agar and then overlaid with potato dextrose
agard (PDA, 1% w/v). After a short drying of the plates, 3 μL of each standardized mold spore suspension (at 10^6 spores/mL) was inoculated onto the center of the plates and then incubated aerobically at 30°C for 5–10 days. The diameter of single mold colonies was measured and compared with control samples in which the Lactobacillus cultures were replaced by MRS broth. The results are summarized in Fig. 18.6. A strain-specific inhibitory activity against molds (from the species A. niger, A. awamori, T. viride, and T. harzianum) was observed. The most promising results were demonstrated by KR51 and KR53, which showed complete inhibition of all test-mold growth (Fig. 18.6).

In addition, a confirmation of these promising results with MRS cultures of LAB was obtained with a new in vitro agar diffusion method—monolayer agar plate assay (Fig. 18.6B). This model system facilitates the contact (direct interaction) between tested LAB strains and the target fungal cultures and makes it easy to estimate the inhibitory effects in vitro and in situ.
18.4.4 MONOLAYER AGAR TESTS WITH 72-H ULTRASONICATED LAB CULTURES

The inhibitory activity of *Lactobacillus* against molds can be established at different times and is caused by different factors. The early phase is registered during fermentation and characterized by the production of compounds such as organic acids. The late phase, which occurs at the end of cell growth, arises from the release of peptidic compounds (Coloretti et al., 2007; Chiavari et al., 1998). It is a common characteristic of all the strains as a physiological consequence of cellular autolysis. Our test
allowed the comparison of antifungal effects of the late phases of LAB growth (72-h ultrasonicated cultures) with that of exponential cultures (early phases of growth). With this aim an original protocol has been designed. Briefly, each late-exponential LAB culture (72-h culture) was ultrasonicated (disintegrated), i.e., LAB cells were subjected to cell lysis and then deep frozen. After defrosting the samples, a monolayer was prepared from a mixture of PDA and ultrasonicated LAB samples (1:1 v/v) with 1% (w/v) agar concentration. After a short drying of the plates, 3 μL of each standardized mold spore suspension (1 × 10^6 spores/mL) was inoculated onto the center of the plates and then incubated aerobically at 30°C for up to 5–10 days. The diameter of the single mold colonies was measured and compared with control samples. Aliquots from *Lactobacillus* lysates were replaced by an equal volume of MRS broth as a control (Fig. 18.7). A strain-specific inhibitory activity against molds (*A. niger*, *A. awamori*, *P. claviforme*, and *T. viride*) was observed. In addition, a weaker LAB activity against *A. niger* and *A. awamori* was noted (Fig. 18.7) in comparison with the previous agar tests (Fig. 18.6A and B). All *Lactobacillus* spp. completely suppressed the development of *P. claviforme* (Fig. 18.7). With regard to *T. viride*, our LAB isolates showed the same promising results. Even strains KR4 and KR51 fully inhibited this test mold (Fig. 18.7). The most promising results were demonstrated by *L. brevis* KR4 and *L. brevis* KR51. Their inhibitory effect is probably because of the peptides produced in the late stages of growth and released after autolysis. The inhibitory activity in the late (postfermentative) phase, as presented in some *Lactobacillus* strains, has interesting technological possibilities for application in the production of a great number of fermented food products, for example, dry fermented sausages and cheeses with a long stage of maturation. The summary of the observed results proved a strain-specific production of acidic and/or proteinaceous metabolites synthesized during the different phases of the fermentation process.

**18.4.5  ANTIFUNGAL ACTIVITY—MILK SAMPLES**

All in vitro tests showed a broad and highly promising potential of the newly characterized strains from katak. Antifungal activity of the four *L. brevis* KR strains against test culture representatives of carcinogenic, toxigenic, deteriorative, and allergenic fungi from the genera *Aspergillus*, *Fusarium*, *Penicillium*, and *Trichoderma* was estimated in a modified protocol of the agar-layer diffusion method. With high practical potential overnight, *L. brevis* KR cultures, cultured in skimmed milk, were mixed with temperate PDA agar at 45°C in equal volumes and poured into plates. This step was followed by the addition of the mold spores. In this study, strains *L. brevis* KR3, KR4, and KR51 completely suppressed the growth of *P. claviforme*, *A. awamori*, and *A. niger*. With regard to *A. flavus* and *T. viride*, a lower and strain-specific inhibitory activity was observed (Tropcheva et al., 2014). The present results, summarized in Figs. 18.6 and 18.7, provide new information on LAB’s biodiversity and biological activity. They are the first published data on the antifungal activity of Bulgarian LAB strains in general and in terms of successful suppression of *A. flavus* and *Trichoderma* species by *L. brevis* (Tropcheva et al., 2010, 2014).

The presented data on the antagonistic activity of newly isolated *Lactobacillus* strains is an important step in the characterization of autochthonous microbiota of katak. The *L. brevis* KR from ahtchak and S1–S4 strains from krutmatch possess promising and desirable properties, suggesting their potential applications in different food technologies. However, there is a need for more experiments to be conducted to elucidate the nature and mechanisms by which the reported antifungal effects were accomplished and therefore the possibility for their practical use.
18.5 CONCLUSION

Katak is a unique fermented product with great traditions in Bulgarian dairy production. The combination of specific LAB and adapted traditional technologies to the geographical regions plays a central role in the preparation of different appetizing products, with original organoleptic properties (from yogurt-like to cheese-like taste and structure) and prolonged shelf-life (up to 12 months). Katak’s autochthonous microbiota, presented mainly by Lactobacillus species, possesses promising bioprotective potential. However, further assessment of functionality and technological relevance of these newly isolated LAB is a promising tool for the development of new starters/adjuncts and is still in progress. This should be the science-based contemporary model for the collection of new potential protective LAB strains, which could be applied in the market of functional foods and/or in medicine. The present work confirms the fact that the well-conserved niches, such as traditional fermented dairy products, collected from different ecologically pure regions are a promising source of new active LAB strains.

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- Dr. D. Nikolova and Dr. Y. Evstatieva from the Biotechnology Department of the Biological Faculty, Sofia University, for test cultures of molds.

REFERENCES


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19.1 INTRODUCTION

Fermented milk and yogurts are products very appreciated in Brazil, from infancy to old age. This popularity is due to several factors, including their pleasant sensory properties and practicality, being ready-to-eat products. In addition, their nutritional qualities, especially in relation to calcium content and proteins of high biological value and higher digestibility (lactose is partially or fully hydrolyzed by the active enzyme lactase from microorganisms), lead to wide consumer acceptance.

Under Brazilian law, yogurt is necessarily obtained by fermentation of milk by the cultures *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*, which need to be viable and abundant in the final product. Products obtained using other cultures, such as *Lactobacillus casei*, *Lactobacillus acidophilus*, or *Bifidobacterium* spp. are called fermented milk products rather than yogurt. Yogurts or fermented milk submitted to heat treatment after fermentation process are not marketed in Brazil. However, the legislation allows the commercialization of whey beverages to be heat-treated after fermentation (Brasil, 2005, 2007a).

Yogurt is considered a high income-elasticity product and its consumption in Brazil is strongly associated with the current economic conditions, since consumption increases very significantly (until four times) when the economy is favorable, reaching 13 kg/year per capita in stable economic times. This is similar to the per capita consumption of the neighboring country Argentina, with 15 kg/year in the same period (Moraes, 2015a). However, the average consumption in Brazil is 7.5 kg/year. For some Brazilian dairy companies, yogurt is the core business, and many new options have been launched in the stable economy period, such as probiotic yogurt, sundae-type yogurt, Greek yogurt, lactose-free yogurt, organic yogurt, goat milk–based yogurt, salty yogurt, and flavor innovations.

Also, yogurts and fermented milk in Brazil tend to be consumed at breakfast or accompanied by breakfast cereals, fruits, honey, various types of nuts and walnuts, or even in the afternoon as a snack. In the case of fluid yogurts, there is no need to eat them with a spoon. Another possibility is their use in culinary recipes to replace ingredients with higher fat content (as fatty cheese), obtaining low-calorie preparations. For this type of application, in 2015, salty yogurts appeared on the market, which can be used either in cookery or eaten with cheese and other accompaniments (Fisberg et al., 2014; Moraes, 2015b).
Yogurts and other types of probiotic fermented milk entered the market with strong advertising campaigns with healthy claims, with special focus on intestinal motility, and gained market quickly. Many people became regular consumers of these products, which began to be prescribed by professional health care providers for therapeutic purposes.

A striking difference between the yogurts produced in Brazil when compared to other countries is the fact that they are less acidic and sweeter, which comes with the Brazilians’ preference for higher levels of sugar in food products.

The unflavored yogurts, called *natural yogurts* in Brazil, meet consumers’ interest in products containing lower amounts of additives, such as potassium sorbate and flavorings, and the organic yogurts stand out within this public awareness of health issues. It is worth noting the differences in formulations in Brazil, when compared to other countries—Brazilian formulations usually contain more ingredients and higher sugar levels. Yogurt produced by a multinational company in Brazil, for example, has double the amount of ingredients and additives than its European version. Given the large number of additives used by some brands, yogurts are erroneously considered by some consumers as ultraprocessed products, which lead to rejection by some people who prefer to prepare the product at home or buy brands with a lower content of ingredients as unflavored organic products (*Proteste, 2015*).

New from the Brazilian dairy sector are two products: night yogurt that is intended for physical therapy purposes to aid in muscle recovery (being richer in protein), and milk fermented by the probiotic yeast *Kluyveromyces marxianus*, which is regulated by the National Health Surveillance Agency (ANVISA).

Whey beverages are products ready for consumption, formulated with milk or dairy products, other ingredients (sugar, sweeteners, thickeners, flavorings, fruit concentrates) and whey (liquid, concentrate, or powder). They can be divided into two broad categories: fermented and nonfermented whey beverages. Among the nonfermented category, the most popular is the chocolate beverage. Fermented whey beverages are very similar to yogurt and fermented milk, which can lead to confusion for the consumer, making it difficult for them to understand the products and their specific differences.

Yogurt is the basis for the production of fermented whey beverages. Therefore, the difference between the fermented whey beverages and fermented milk and yogurts lies in the fact that for fermented whey beverages the use of vegetable fat is allowed, whey is a mandatory ingredient, and a lower proportion of milk basis (at least 51%) and milk protein levels (ranging from at least 1.2 to 1.7 depending on the product type) is allowed (*Brasil, 2005*). Permission to use vegetable fats has given rise to development of new products containing oils, which can be significant sources of fatty acids with health benefits. Due to the presence of whey in their composition, these products cost less and therefore are attractive to low-income consumers.

This chapter will focus on the production of fermented milk, yogurts, and whey beverages in Brazil, focusing on the regulatory aspects, consumer market, processing steps, and products available in the market, including those containing functional ingredients (probiotic and prebiotic), or for particular nutritional uses (lactose-free and light and diet formulations).

19.2 MARKET OF FERMENTED MILK, YOGURTS, AND WHEY BEVERAGES

Brazil produced around 954,000 tons of fermented dairy products in 2014. Yogurts and fermented milk made up 75% of the total, while the fermented whey beverages corresponded to 25%. *Fig. 19.1* shows the percentage of sales for each category.
Yogurt is the best selling of the category, and leads consumers’ preference. The products in larger packages (1 L) stand out for their more affordable price and focus on family consumption and breakfast, which represent 35% of sales. The second option (28%) is the stirred yogurt with fruit pulp, followed by fluid yogurts in individual portions (12%), fermented milk (10%), natural yogurts (8%), and Greek yogurts (7%).

Yogurt consumption has been growing at an average rate of 5% per year, probably due to the increased purchasing power of the population and/or the introduction of sweetened and/or fruit-flavored formulations in the market. It is known that not all consumers appreciate yogurt in its natural form, thus the addition of fruit leads to an improvement in the sensory characteristics, and attenuates the sour taste of fermented products. Among the yogurt flavors, strawberry represents 70%–80% of the volume produced in the country (Oliveira, 2009).

However, Brazil still has a low yogurt per capita consumption, which is around 7.5 kg/year, compared to 15 kg for Argentines, 20 kg for Portuguese, and 37.2 kg for Dutch and French (Moraes, 2015a). It is believed that higher investments in sensory analysis by Brazilian industries could increase the consumption of yogurts and fermented milk, focusing on the optimal sensory profile aimed to obtain a product with flavor and texture consistent with the desires of Brazilians. In addition, the price of yogurt in the country is very high, which discourages consumption by a large portion of the population.

Studies have shown that 68% of consumers buy yogurt at least once a month, while only 25% buy it between one and five times per week. Considering only classes A and B, categories with higher acquisitive power, 79% of people consume yogurt, of which 60% eat it at least once a week.

In terms of innovation, Greek yogurt boosted sales in recent years. Part of the success of this type of yogurt lies on its characteristic of being considered natural in addition to having more consistent and creamy texture, which is intermediate between the set and fluid yogurts. In Brazil, this product contains 10%–30% fat and 5%–10% protein, depending on the brand. Although the total sales are still low when compared to fluid yogurts, Greek yogurt generates more profit for industries with respect to the production costs and sales value.
In turn, the penetration of whey beverages in Brazil is nearly 50%, and the highest consumption is during breakfast. The higher consumption is observed for females (56%) when compared to males (44%). Children aged 1–11 years are the biggest consumers, consuming 47% of the total volume produced.

19.3 GENERAL ASPECTS OF FERMENTED MILK AND WHEY BEVERAGES

The processes for producing fermented milk, yogurt, and fermented whey beverages are very similar, and can be summarized in the following sequence: standardization of milk solids (when necessary) or mixture of liquids and dry ingredients, heat treatment, cooling, inoculation using specific microorganisms, incubation, cooling, handling, and packaging. In the case of nonfermented whey beverages, there is no fermentation process, and the products have very different characteristics (Chandan and O’Rell, 2006; Lourens-Hattingh and Viljoen, 2001). Fig. 19.2 shows the main differences between yogurts, fermented milk, and fermented whey beverages.

Table 19.1 shows the average nutritional value of yogurts, fermented milk, and whey beverages available in the Brazilian market. There is a difference in the chemical composition of the various products. Although all of them have health benefits, yogurt and fermented milk have gained nutritional

FIGURE 19.2

Main characteristics of yogurt, fermented milk, and fermented whey beverage.
Table 19.1 Chemical Composition and Caloric Value of Yogurts, Fermented Milk, and Whey Beverages Available in the Brazilian Market

<table>
<thead>
<tr>
<th>Component</th>
<th>Fermented Milk</th>
<th>Set Yogurt</th>
<th>Stirred Yogurt With Strawberry Pulp</th>
<th>Strawberry-Flavored Fluid Yogurt</th>
<th>Greek Yogurt</th>
<th>Strawberry-Flavored Fermented Whey Beverage</th>
<th>Chocolate Whey Beverage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caloric value (kcal/100 g)</td>
<td>64</td>
<td>59</td>
<td>116</td>
<td>83</td>
<td>151</td>
<td>76</td>
<td>93</td>
</tr>
<tr>
<td>Carbohydrate (g/100 g)</td>
<td>14</td>
<td>6</td>
<td>22</td>
<td>16</td>
<td>16</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>Protein (g/100 g)</td>
<td>2</td>
<td>4</td>
<td>2.5</td>
<td>2.6</td>
<td>5.1</td>
<td>1.8</td>
<td>1.7</td>
</tr>
<tr>
<td>Total fat (g/100 g)</td>
<td>-</td>
<td>2</td>
<td>2.4</td>
<td>1.2</td>
<td>7.5</td>
<td>1.1</td>
<td>2.7</td>
</tr>
<tr>
<td>Saturated fat (g/100 g)</td>
<td>-</td>
<td>1.5</td>
<td>1.5</td>
<td>0.8</td>
<td>5.1</td>
<td>0.7</td>
<td>1.2</td>
</tr>
<tr>
<td>Fiber (g/100 g)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Calcium (mg/100 g)</td>
<td>86</td>
<td>147</td>
<td>150</td>
<td>94</td>
<td>203</td>
<td>49</td>
<td>61</td>
</tr>
<tr>
<td>Sodium (mg/100 g)</td>
<td>38</td>
<td>62</td>
<td>41</td>
<td>41</td>
<td>42</td>
<td>41</td>
<td>80</td>
</tr>
</tbody>
</table>

*Data from the label of Brazilian products.*
prominence by containing the largest milk basis. The advantages include greater amounts of protein and calcium, reduction of the symptoms of poor digestion, and improvement in intestinal microflora when containing probiotic cultures, which are microorganisms capable of colonizing the intestine, resulting in reduction of colonization by pathogenic microorganisms.

Whey proteins have interesting characteristics, such as favoring the immune system of the individual and presence of compounds that assist in digestion. Whey beverages have lower caloric value in some cases. Therefore, the consumption of whey beverages should not be disregarded, since they are a more affordable alternative to consumers with less purchasing power.

### 19.4 FERMENTED MILK AND YOGURT

According to the Brazilian law, fermented milk products comprise a series of products such as yogurts, fermented or cultured milk, acidophilus milk, kumys, and fermented curd, among others (Brasil, 2007a). However, in the country, there is a significant market only for fermented milk and yogurts.

Different microorganisms can be used for the production of fermented milk. The most common bacteria are *L. acidophilus*, *L. casei*, *Bifidobacterium* sp., *S. thermophilus*, and/or other lactic acid bacteria, which contribute to the characteristics of the final product.

Yogurt is the best-known fermented milk product and is widespread in the country. It is a thick gel, white and slightly acidic, very nutritious, available in natural form or mixed with fruit preparations. For yogurt manufacture, two types of microorganisms must be used, *L. bulgaricus* and *S. thermophilus*, which can be accompanied or not by other cultures. This definition makes clear that products that do not contain these two microorganisms are classified as fermented milk products rather than yogurts.

Brazilian law has established minimum counts of specific microorganisms throughout the shelf life of the product, namely: $10^7$ cfu/g in yogurts, and $10^8$ cfu/g in fermented or cultured milk (Brasil, 2007a). In the case of yogurts and fermented milk with mixed strains, counts refer to the total lactic acid bacteria counts.

Fermented milk products and yogurts may be classified according to the presence or absence of nondairy ingredients, chemical composition, and flavoring agents (Tamime, 2002a,b; Brasil, 2007a). According to addition or not of nondairy ingredients, fermented milk products and yogurts can be classified as follows:

- **With no addition.** Only milk and other products of milk origin are used in the manufacture. The milk basis is 100% (w/w) of the total ingredients.
- **With addition of sugar, sweeteners, and/or flavorings.** Optional ingredients should be exclusively sugars, with or without carbohydrates (except polyalcohol and polysaccharides) and/or starches, modified starches and/or maltodextrin, and/or flavorings.
- **With addition.** The product contains dairy and nondairy ingredients. The milk basis is at least 70% (w/w) of the total ingredients.

With respect to flavor, yogurt and fermented milk can be classified into natural, with fruits, or flavored with addition of flavorings. Based on the chemical composition, yogurt and fermented milk can be classified according to the fat content as: creamy (at least 6 g fat/100 g); whole (minimum 3 g fat/100 g); partly skimmed (maximum 2.9 g fat/100 g); and skimmed (maximum 0.5 g fat/100 g). Legislation also requires that the acidity of fermented milk should be between 0.6% and 2% lactic acid, while in yogurts it may range between 0.6% and 1.5% lactic acid, and the protein content should be above 2.9/100 g for the product without addition of nondairy ingredients (Brasil, 2007a).
Yogurt has another classification based on the physical characteristics of the gel, and is divided into three types:

1. Traditional, classic, or set yogurt. The fermentation process takes place within the package. The product does not undergo homogenization after fermentation, resulting in a firm and consistent product. It should have sufficient consistency to be eaten using a spoon.

2. Stirred yogurt. The fermentation process takes place in tanks or incubators with subsequent breakdown of the gel, and packaging. The semisolid texture is obtained upon cooling the product, and thickeners can be used.

3. Liquid or fluid yogurt. The fermentation process is carried out in tanks, followed by breaking of the gel and homogenization, acquiring liquid consistency. It is marketed in plastic containers like bottles or cartons.

Fermented milk and yogurts without sugar addition (diet), with reduced sugar (light), lactose free, fortified with minerals and/or vitamins, or containing functional compounds, such as probiotics and prebiotics are also marketed.

Fig. 19.3 describes the overall manufacture of set, stirred, and fluid yogurts. The fermented milk production process follows the same steps, being found mostly in fluid form.

19.4.1 RAW MATERIALS

In Brazil, almost all types of fermented milk and yogurts are made from bovine milk. However, a few types of yogurts with buffalo, goat, or sheep milk are already available on the market.

Milk must be fresh, produced under optimum sanitary conditions, with low spoilage microorganisms, somatic cell counts and mycotoxins, and absent of pathogens and inhibitory compounds, such as antibiotic residues, pesticides, or sanitizers. Once the fermented milk and yogurts are products that require the action of lactic acid bacteria, one or more of the herein-mentioned factors can affect the fermentation process, causing inhibition of the culture, affecting the quality parameters of the fermented products.

Milk for yogurt and fermented milk production should be previously pasteurized and cooled, but it must undergo heat treatment after the addition of sugars, thickeners, milk powder, whey powder, protein concentrates, or other ingredients, aimed to destroy pathogenic microorganisms and make the environment more favorable to the development of the starter culture.

19.4.2 STANDARDIZATION OF FAT AND NONFAT SOLIDS CONTENT

Fat and nonfat solids are adjusted to accomplish the laws of the country and/or meet the desired characteristics of the final product. Fat content is standardized according to the type of product (creamy, whole, semiskimmed, or skimmed).

The nonfat solids of milk comprise lactose, proteins, and minerals, at a concentration from 8.5 to 9 g/100 g milk. Brazilian law does not include this parameter with respect to fermented milk and yogurts, but other countries, such as the United States, have set a minimum level of 8.25 g/100 g. The establishment of minimum values aims to protect consumers by ensuring the maintenance of nonfat solids content in fermented milk and yogurts similar to those found in milk.
The nonfat milk solids content in milk is enough for the preparation of fluid yogurt and fermented milk, since the goal is obtaining a liquid product. In the case of set and stirred yogurts, an increase in nonfat solids is needed. To increase nonfat solids, milk can be boiled until it is reduced by about two-thirds of its volume, through concentration by membrane filtration or addition of skim milk powder. The addition of skim milk powder is the main method used to increase the nonfat milk solids because it provides the ratio of protein/total solids close to that found naturally in milk. Other ingredients may also be used, such as whey milk powder, whey protein concentrate, caseinate, or butter-milk powder. The choice of the method will depend on the availability of materials, cost, and scale of production.

The physical properties of yogurt and the gel consistency are of great importance; generally, the higher the nonfat solids, the higher viscosity and consistency of the final product. Furthermore, the increase in nonfat solids diminishes the tendency to syneresis, and slightly reduces the acid production during fermentation, obtaining a product with lower acidity, which is better accepted by Brazilian
consumers. Commercial yogurts generally have 14%–16% total solids (including the nonfat solids); therefore, the addition of nonfat solids at a concentration of 2%–4% is performed by dairy industries.

19.4.3 **ADDITION OF SUCROSE AND/OR SWEETENERS AND OTHER INGREDIENTS**

Sucrose addition in fermented milk products and yogurts with fruits, fruit flavored, or naturally sweetened is carried out before the heat treatment, ensuring the decrease of spoilage microorganisms that may be present in this ingredient. In Brazil, the use of sugar can be considered excessive, and can reach 12%–15% in the formulation. Consumers concerned about health have sought yogurts and fermented milk with reduced or no sugars, light yogurts, or sugar-free yogurts, with sucrose replaced by other sweeteners.

It is worth mentioning that the sugar addition can lead to inhibition of lactic cultures, resulting in longer fermentation times and a decrease in acidity. This inhibition can be due to the adverse osmotic effect of milk solutes and lower water activity of the medium.

Additives such as gelatin, starches, gum (carrageenan, locust bean, guar, tragacanth, arabic, acacia, xanthan, karaya, gelan, konjac), alginates, microcrystalline cellulose, carboxymethylcellulose, and pectins, among others, are permitted by legislation as thickeners and stabilizers only in nonfat fermented milk and yogurt, or products with addition of nondairy ingredients. For fermented milk products and yogurts with no additions, the use of additives is not permitted (Brasil, 2007a).

These additives improve the appearance and texture of the products. Gelatin is effective for moisture control and improved texture, without interfering in the taste of the product. Low-methoxyl pectins are used in stirred yogurts to promote increase in viscosity after breaking the gel, whereas high-methoxyl pectins have the function of stabilizing fluid yogurt. For pectins and gelatins, the maximum allowed limit is 10 g/kg, while 5 g/kg can be used for the other additives, singly or combined (Brasil, 2007a).

Optional ingredients can also be used, including milk concentrate, cream, butter, anhydrous milk fat or butteroil, milk powder, food caseinates, dairy proteins, other solids from milk origin, whey, whey concentrate, maltodextrins, honey, coconut, cereals, vegetables, dried fruits, chocolate, spices, and coffee, among others. Starches and modified starches are allowed in a maximum concentration of 1% (w/w) of the final product. Optional nondairy ingredients, alone or in combination, should be present in a maximum concentration of 30% (w/w) of the final product classified as fermented milk or yogurt with addition (Brasil, 2007a).

19.4.4 **HOMOGENEIZATION**

Homogenization affects milk fat globules, thus it is very important in fermented milk products and yogurts made with whole milk. Although milk fat does not directly participate in gel formation, the reduction in size and increase in number of fat globules as a result of homogenization modifies the gel formed, increasing the viscosity. Furthermore, there is lower syneresis, with homogeneous dispersion of the constituents, improving flavor, consistency, performance, and digestibility of the product.

This process also changes the structure of milk proteins, mainly due to the heating and mechanical stress imposed. Although a significant change in gel formation may not be observed, the curd formed may be slightly less firm than the conventional.

Homogenization is not a mandatory practice and requires relatively expensive equipment; it is used almost exclusively in industries with large production.
19.4.5 **HEAT TREATMENT**
Heat treatment aims to eliminate vegetative forms of pathogenic microorganisms; to eliminate or reduce spoilage microorganisms to an acceptable number; to reduce the total microbial population that can interfere with development of lactic bacteria; to denature whey protein to improve product’s texture, prevent syneresis during storage; and to hydrate stabilizers after heating.

Different temperatures can be used in the manufacture of fermented milk and yogurts, such as 85°C for 30 min, 90–95°C for 5 min, or 105°C for 10 s (ultrahigh temperature; UHT). In the case of batch processes, lower temperatures are used (85°C), while intermediate temperatures (90–95°C) are used in continuous processes in heat exchangers. The use of UHT does not provide the same benefits and physical properties necessary for microbial growth and development of texture. The time/temperature binomial of 85°C for 30 min is recommended, once it results in a final product with better texture characteristics due to changes caused by denaturation of casein and whey proteins.

19.4.6 **DECREASE IN TEMPERATURE**
After heat treatment, milk should be subjected to adequate temperatures prior to inoculation of the lactic acid culture. Suitable temperatures for fermented milk and yogurts are quite different, ranging from 37°C to 40°C and 42°C to 45°C, respectively. Higher temperatures can inhibit or, in extreme cases, eliminate the microorganisms from the culture. On the other hand, very low temperatures prolong the fermentation time.

19.4.7 **INOCULATION AND FERMENTATION PROCESS**
The lactic acid culture is generally used at a concentration of 1%–2% (v/v) with slow homogenization to uniformly distribute the microorganisms into the medium. It is commercialized in liquid, frozen, or dehydrated forms. Liquid culture is grown in sterilized reconstituted skim milk and stored under refrigeration (8°C) for a period of less than 2 weeks. The frozen culture may be stored for 3–6 months, while the dehydrated cultures (vacuum, spray-dried, or lyophilized) can be stored for more than 6 months.

Lyophilized cultures are the most marketed, because they are in powder form for direct use, have good standardization, and do not require activation, dispensing subcultures and reducing labor and risk of infection by phages.

For set yogurt, fermentation occurs within the package, while fermentation tanks are used for stirred yogurt, fluid yogurt, or fermented milk.

The role of all cultures is to produce lactic acid by the fermentation of lactose in as short a time as possible so that milk with a pH from 6.4 to 6.7 reaches pH 4.5–4.6 to obtain the fermented product. Furthermore, the starter cultures contribute to several factors, including the production of flavor compounds and other metabolites, resulting in a product with sensory characteristics desired by the consumer; improvement of the nutritional value with the release of free amino acids or vitamin B synthesis; and development of the product’s texture, besides the health benefits due to the beneficial viable cells during consumption.

Starter cultures can contain one or more species. Those that contain a single strain are called pure cultures; those that have two or more species are called multiple cultures; and those with indefinite strains, such as natural strains, are called mixed cultures.
Lactic acid cultures can also be classified into homofermentative or heterofermentative strains. Homofermentative strains almost exclusively produce lactic acid, while the heterofermentative strains produce lactic acid and other compounds such as CO\textsubscript{2}, ethanol, or acetic acid.

For yogurts, starter cultures comprise two specific microorganisms \textit{Lactobacillus delbrueckii} ssp. \textit{bulgaricus} and \textit{S. thermophilus} growing symbiotically. \textit{S. thermophilus} produces lactic acid that lowers pH to an optimum value for \textit{L. delbrueckii} ssp. \textit{bulgaricus} growth, releasing formic acid and sulfur dioxide, which stimulate growth of the latter. In turn, \textit{L. delbrueckii} ssp. \textit{bulgaricus} releases amino acids and peptides from milk proteins, stimulating \textit{S. thermophilus} growth. However, this symbiosis requires cultivation in an \textit{S. thermophilus}/\textit{L. delbrueckii} ssp. \textit{bulgaricus} ratio of 1:1; 2:1; or 3:2. In Brazil, cultures with lower \textit{L. delbrueckii} ssp. \textit{bulgaricus} counts or even without this microorganism have been used, such as commercial ABT culture (\textit{L. acidophilus}, \textit{Bifidobacterium}, and \textit{S. thermophilus}), aiming to reduce the postacidification, resulting in less-acidic products for a longer period of time. Importantly, however, the absence of \textit{L. bulgaricus} impairs the classification of the product as yogurt, which is therefore considered as fermented milk.

The optimal growth temperature for yogurt bacteria is between 42°C and 43°C, since the generation times of both microorganisms are similar at this temperature. At higher temperatures (45°C) there is a predominance of \textit{L. delbrueckii} ssp. \textit{bulgaricus}, while \textit{S. thermophilus} is favored at lower temperatures (37°C).

In the case of fermented milk, pure cultures (\textit{L. acidophilus}, \textit{L. casei}, \textit{Bifidobacterium} sp., \textit{S. thermophilus}) are used, at 37–40°C, which is the optimal temperature range for the majority of microorganisms.

### 19.4.8 Cooling

The resulting gel is subjected to cooling from the moment it reaches the desired acidity for the product. The degree of acidification depends on the type of product, cooling method, and desired acidity. Generally, at this time, fermented milk and yogurt have pH between 4.5 and 4.6 with fermentation times between 4 and 5 h or longer for fermented milk products, depending on the culture used.

The cooling temperature of 15–20°C must be reached within an hour to an hour and a half. Cooling has the role of reducing the metabolic activity of the culture, controlling the acidity of the product, and preventing postacidification.

For the set yogurt, cooling can be achieved by spraying cold air in the incubation room or by transferring the packages to air-conditioned rooms (2–4°C). For stirred and fluid yogurts, which are fermented in tanks, cooling is done by circulating cold water (2°C) on the equipment wall.

### 19.4.9 Breakdown of the Gel

This step is not carried out in set yogurt. In stirred yogurts, the time and stirring rate should be standardized to obtain products of similar consistency at the end of the process. In the case of fluid yogurts and fermented milk, it is important to satisfactorily homogenize the gel, eliminating large pieces or flakes, and reducing the viscosity to a minimum level. Also, during the breaking of the curd, various ingredients can be added, such as colorants, flavors, pulps, and, others.
19.4.10 ADDITION OF FRUIT PREPARATIONS

The addition of fruit preparations is a trend in the yogurt market to meet the demand of consumers who prefer flavored yogurts rather than the natural ones. In addition, this may be a strategy of the industry to mask acidity of products through flavorization.

Flavorization is done after fermentation, and is a critical control point. Thus, it is fundamental to choose raw materials with guaranteed quality to minimize the risk of contamination.

Fruit preparations contain fruit, fruit components, sugar, extracts, flavorings, colorants, stabilizers, and acids. They are preserved by chemical or heat treatments. The fruit concentration varies with the type of fruit and type of product, and can contain fruit pieces, fruit juice, fruit jellies, or several ingredients (cereals, honey, vegetables, etc.). In general, fruit preparation with 20%–40% soluble solids, 35–65°Brix, and pH 3–4.5 can be used, comprising up to 5% in the formulation of products. However, higher concentrations may be added provided that the yogurt formulation before fermentation contains milk solids (in particular milk proteins) to compensate for the addition of larger fruit pulp contents. Current legislation permits the presence of sorbic acid and its sodium, potassium, and calcium salts at a maximum concentration of 300 mg/kg in the final product.

19.4.11 BOTTLING AND STORAGE

Stirred yogurt should be stored under refrigeration for over 24 h before being sold, so that there is a complete maturation, avoiding texture defects. The most popular package is the individual portion of 125–150 g, while in Brazil, there are portions of 80–250 g. The packages are preformed, filled, and heat-sealed with foil seals or caps.

Variations in temperature during storage can change texture and viscosity, resulting in syneresis, and favoring the development of spoilage microorganisms. Furthermore, exposure to higher temperatures can accelerate biochemical reactions such as lipid oxidation and increase hydration of the protein products, dehydration of the surface, and changes in color of the fruit. Therefore, during storage, the temperature should be kept between 2°C and 5°C, and never exceed 10°C in the intermediate stages of the distribution chain. Overall, the products have a shelf life of 35 days.

19.5 GREEK YOGURT

In Brazil, firmer yogurts are called “Greek yogurt.” It is worth mentioning that the product is “Greek” in name only, because it does not contain ingredients used in Greece; it only alludes to be a more consistent and tastier product, as the product originally produced in the Balkans. This name refers to a marketing strategy to boost sales (Estadão, 2014).

The first release of Greek yogurt in Brazil was in 2012, by a traditional company. After that, all the other leading companies in the dairy market launched similar products. Consumers agree to pay more (even double the value of a conventional yogurt), once it is a product with higher value, and gourmet appeal.

In Brazil there is no specific identity standard in legislation for this type of product. Although Greek yogurt can be obtained by concentration of yogurt components by membrane technology (ultrafiltration) or centrifugation (after fermentation), the most widely used process is the direct addition of proteins and stabilizers before fermentation (simpler method that does not require specific equipment). In
As Greek yogurt has considerably higher solids content when compared to traditional yogurt and fermented milk, changes in behavior of certain types of lactic culture may occur. In the traditional process of manufacturing the Greek yogurt, the starter bacteria produce lactic acid in milk before concentrating it by centrifugation or bags (Berge system). Then, other ingredients are added to the concentrated fermented milk.

When the solids content is increased in the formulation before fermentation, it is necessary to fit the type of culture, dosage, fermentation time, among other parameters, once cultures and fermentation conditions used to manufacture the traditional yogurt cannot produce Greek yogurt with appropriate characteristics. Among the changes, the unwanted production of exopolysaccharides due to the stress of microbial cells stands out.

Greek yogurts produced in other countries are obtained by concentration of the ingredients followed by serum separation, which results in products with higher protein content. In Brazil, consumers have a perception that the product is protein rich; however, some products have higher fat rather than protein, as can be seen in Fig. 19.4.

As lactose-free products are a trend in dairy products, Greek yogurt has also gained lactose-free versions. Low-calorie Greek and Greek fluid yogurts are other recent launches in Brazil.

**19.6 SUNDAE-TYPE YOGURTS**

Sundae-type yogurt follows the manufacturing steps of set yogurt, differing in the addition of pulp. The pulp is added first to the package, followed by the milk basis consisting of milk, milk powder, sugar, stabilizer/thickener, and/or preservatives. After that, the fermentation process is performed. In the Brazilian market, the terminology “sundae-type yogurt” has not been explored, but its concept was naturally embedded among consumers (Moraes, 2015d). Another version of sundae-type yogurt is
found in Brazil, wherein the package has two compartments and yogurt and fruit pulps are mixed when the folding package is opened and the consumer turns the fruit pulp onto the yogurt.

Before the invention of sundae-type yogurt, some consumers were used to making it at home, by purchasing unflavored yogurt and adding fruit jellies. It is worth noting that unflavored yogurts were marketed without the addition of sugar until an update by the Brazilian legislation that allowed the marketing of sweetened products. Thus, the addition of fruit jellies and honey also represented a way to sweeten the yogurt, especially for consumers who made sundae-type yogurt in a traditional manner.

19.7 PROBIOTIC YOGURT AND FERMENTED MILK

Yogurts and fermented milk cover most of the functional foods available in Brazil, probably because milk is a nutritious and natural part of a balanced diet. The functionality concept in dairy products means the enrichment of traditional basis that already has a healthy appeal.

The functional ingredients used in fermented milk products and yogurts include dietary fiber, vitamins and minerals, phytochemicals, bioactive peptides, unsaturated omega-3, probiotics, prebiotics, among others. Fermented milk and probiotic yogurts represent the largest market for functional foods in Brazil.

Probiotics are live microorganisms that confer beneficial effects to the individual when administered in adequate amounts (FAO/WHO, 2001). Many species have been tested as probiotics, consisting of lactic acid bacteria (lactobacilli, streptococci, enterococci, lactococci), bifidobacteria, Bacillus spp., Escherichia coli, and yeasts and mold, such as Saccharomyces spp. and Aspergillus spp. L. acidophilus, bifidobacteria, and L. casei species are the most used in dairy products in Brazil.

Probiotic cultures with good technological properties are those that exhibit good proliferation in milk, remaining viable during storage and providing acceptable sensory characteristics.

Although the microorganisms used in yogurt manufacture (L. delbrueckii ssp. bulgaricus and S. thermophilus) can promote health benefits to consumers, they are not able to survive the acidic conditions and bile salt concentrations commonly found in the gastrointestinal tract, not meeting the selection criteria for potential probiotic strains. Thus, not all yogurts can be considered as probiotic products.

To have a positive health impact, probiotic counts must reach adequate levels in the product, generally from $10^6$ to $10^9$ cfu/mL or g. In Brazil, the minimum viable counts should be in the range of $10^8$–$10^9$ cfu in the daily serving of a particular food containing probiotic strains (Brasil, 2007b). Lower values can be accepted since the company proves its effectiveness in lower concentrations than that established by law.

Traditional manufacturing procedures have been used for the production of fermented milk and probiotic yogurts, except the incorporation of probiotic cultures. Homogenized and heat-treated milk is added milk powder or other ingredients to adjust the dry matter and inoculated with lactic acid bacteria and/or probiotic cultures. Then, incubation is carried out until the pH reaches 4.5–4.6, using temperature ranges of 37–40°C and 40–45°C for fermented milk and yogurts, respectively.

Probiotic cultures can be added to yogurt by four different ways: at the beginning of fermentation (as a component of the lactic acid culture, or by direct addition of concentrated culture); after fermentation; by double fermentation (probiotics fermented separately for 2 h at 40–42°C and then added to milk containing starter cultures, with further fermentation until the desired pH); or in separate tanks (probiotics and starter cultures grown separately and then joined).
The addition of probiotic cultures before fermentation has the advantage that the culture is more adapted to the environment, since it participates in all of the process changes, including the gradual decrease in pH. Moreover, probiotics use the peptides released from starter cultures during fermentation. From the manufacturer’s point of view, this is the most practical alternative, compatible with industrial routines.

The use of probiotics after fermentation should be considered only in special circumstances, once probiotics are subjected to a very acidic environment, coming in contact with the metabolites produced by lactic acid culture during fermentation. Thus, probiotics must be added in sufficient amounts to maintain the microbial populations during the shelf life of the product.

Double fermentation is an effective approach because the lactic acid culture is added to milk at the end of the lag phase of probiotics, and during fermentation they can reach the exponential phase, thus reaching high populations. This fermentation condition leads to good viability of probiotic strains and less postacidification of the fermented products.

Fermented milk can be produced with only probiotic microorganisms if proteolytic strains capable of growth in milk are selected. Otherwise, the fermentation time becomes very long, and the sensory characteristics of the product may be compromised. Thus, some strategies have been studied, such as the addition of amino acids, peptides, or micronutrients in milk, or an increase in the solids content.

A preliminary study on the compatibility of ingredients and lactic acid culture is needed for the production of fermented milk and yogurts containing probiotic cultures. Ingredients such as sugar, flavoring, preservatives, fruit juice or pulp, and sweeteners can affect the viability of the probiotic cultures, reducing populations during manufacture or storage. Good compatibility between acid lactic cultures and probiotics can prevent fermentation problems, such as inhibition of probiotic culture by acids, peroxide, bacteriocins, or other metabolites produced by lactic culture.

Attention should be paid to the selection of probiotic cultures, since some strains can negatively affect the sensory characteristics of the processed product. Some Bifidobacterium strains have the ability to produce acetic acid, especially during long fermentation times, yielding products with vinegar flavor. Highly proteolytic probiotic strains can lead to formation of peptides that confer cheese flavor in fermented milk.

To maintain adequate levels of probiotic cultures in the product it is necessary to maintain the pH above 4.6, or select strains that are more resistant to the acidic medium. Additionally, the oxygen incorporation during processing (beating and breaking the gel, pumping and filling) must be prevented, as probiotic cultures used in food have anaerobic or microaerophilic metabolism, thus the presence of oxygen can be a threat to survival of probiotics.

Probiotic yogurt and fermented milk should also be stored under refrigeration, as well as their traditional versions, to ensure high survival rate of probiotics and product stability.

Probiotic cultures have been associated with several health benefits, including reduction in gastrointestinal infections; antimicrobial activity; improvement in lactose metabolism; reduction in serum cholesterol; stimulation of the immune system; antimutagenic, anticarcinogenic, and antidiarrheal properties; improvement in symptoms of irritable bowel syndrome; suppression of infections caused by Helicobacter pylori; and reduction of obesity and atopic dermatitis. However, the health benefits provided by probiotic cultures are specific to each strain, that is, no strain will be capable of providing all reported benefits, and strains of the same species cannot be effective against specific health conditions (Cruz et al., 2011).
Table 19.2 shows the fermented milk and yogurts containing probiotics available in the Brazilian market. In some cases, the identification of probiotic strain and the viable population is not clearly shown on the product label. Considering that the health benefits are specific to each strain and the amount of probiotics is relevant, problems of qualitative or quantitative nature can lead to noncompliance of health benefits and products out of standards required by law. This demonstrates the importance of constant monitoring of these products by health agencies.

In Brazil, only food products with probiotics that have their claim for functional property approved by ANVISA can declare on their labels “The (Type of probiotic) contributes to the balance of intestinal flora. Its consumption should be associated with a balanced diet and healthy lifestyle” (Brasil, 2007b).

19.8 WHEY BEVERAGES

Whey beverages are milk products resulting from the mixture of milk, in liquid or powdered form, and whey (liquid, concentrate, or powder). These products have been developed with the purpose of using whey from the cheese manufacture. The use of whey in beverages is one of the most attractive alternatives for use of whey in foods, due to the simplicity of the process, using the equipment available for
milk processing, with minimal investment. These products have won the market, mainly due to information on the nutritional benefits of whey, lower production costs, and, lower price for consumers.

Whey beverages can be classified according to the addition of nondairy ingredients, heat treatment, or lactic fermentation (Brasil, 2005; Damin et al., 2009; Penna, 2010). According to the addition of nondairy ingredients, they can be classified into:

- Whey beverages with no addition. The product cannot contain vegetable fat, fermented milk, and other milk derivatives. The milk basis is 100% (w/w) of the total ingredients of the product.
- Whey beverages with addition. The product can contain vegetable fat, fermented milk, and other milk derivatives. The milk basis is at least 51% (w/w) of the total ingredients of the product.

According to the heat treatment, whey beverages can be classified into:

- Pasteurized whey beverages. The product is subjected to slow pasteurization (62–65°C/30 min) or rapid pasteurization (72–75°C/15–20 s), cooled between 2 and 5°C, packed, and stored under refrigeration.
- Sterilized whey beverages. The product is packed, subjected to direct or indirect vacuum, and conveniently sterilized by moist heat followed by immediate refrigeration, respecting the uniqueness of each product. Sterilization of the packaged product will comply with different time/temperature binomials, according to the packaging capacity. Storage can be at room temperature.
- UHT whey beverages. The product is subjected to temperatures of 130–150°C for 2–4 s, by continuous stream processing, followed by immediate refrigeration (<32°C), packed under aseptic conditions in sterile, hermetically sealed packaging. Storage can be at room temperature.
- Whey beverage heat-treated after fermentation. The fermented product is subjected to heat treatment.

According to the fermentation process, whey beverages can be classified into:

- Unfermented whey beverage (neutral pH)
- Fermented whey beverage (acidic pH)

Unfermented whey beverages are not subjected to fermentative process; thus they do not contain microorganisms in their composition. They are produced with different flavors and have good nutritional quality, being a healthy alternative as a quick snack for children and adolescents. For their low acidity, they are best suited to Brazilians’ taste; chocolate whey beverage is the most popular unfermented whey beverage, which contains cocoa or chocolate liquor, sweeteners, and ingredients that provide stability and consistency to the product.

Fermented whey beverages are subjected to fermentation process, and present more liquid and refreshing consistence when compared to fluid yogurts, and are well accepted by consumers. They have a lower cost due to the presence of whey in their composition, thus attracting consumers, especially those with less purchasing power.

The major problem is that these whey beverages are displayed on supermarket shelves next to yogurts, without clear information on the label, so they can be consumed as if they were yogurts. Although there is no problem regarding the nutritional aspects, they contain more sugar and additives such as colorants when compared to yogurts. It is also worth noting that the percentage of milk basis required for whey beverages is much lower than that for yogurts, and therefore the amount of protein and calcium in the formulation tends to be lower.
19.8.1 COMPOSITION

The mandatory ingredients of whey beverages are milk (fresh, pasteurized, sterilized, UHT, reconstituted, concentrate, powder, whole, partially skimmed, or skimmed) and whey (liquid, concentrate, or powder). In the case of fermented whey beverages, lactic acid bacteria or fermented milk are also used.

Optional dairy ingredients include cream, milk solids, butter, anhydrous milk fat or butteroil, food caseinate, milk protein, buttermilk, and other products of dairy origin. The nondairy optional ingredients are sugars and/or carbohydrates, maltodextrin, nutritive and nonnutritive sweeteners, fruit pieces, pulp or juice, and other fruit-based preparations, honey, cereals, vegetables, vegetable fats, chocolate, dried fruits, coffee, spices, and other natural food flavorings, starches or modified starches, and gelatin, among others.

The amount of milk proteins in dairy beverages can vary from 1 to 2 g/100 g, depending on type of product. Thus, a product made with nondairy ingredients and whey, with no other sources of milk protein cannot be called whey beverage. To be considered a whey beverage, it should contain milk powder as a source of milk protein.

19.8.2 FERMENTED MILK BEVERAGES

The manufacturing technology of fermented whey beverages is quite simple, being of interest mainly for small dairy industries. There are two main processes used in industry for preparing fermented whey beverages (Fig. 19.5).

Whey used in whey beverages may be in the liquid, concentrated, or powder form. The equipment for whey concentration and dehydration is not available in small- and medium-sized dairy industries, needing to be imported. Therefore, the use of whey in liquid form can reduce costs of raw materials and final products.

Liquid whey must have maximum acidity of 13°D, with no curd particles. Thus, it is subjected to filtration or clarification. The characteristics of the other ingredients used in the formulation (milk, hydrocolloids, fruit-based preparations, sugar and sweeteners, flavorings, colorings, and conservatives) are similar to those permitted in yogurts and fermented milk products.

The first manufacturing process is quite similar to the manufacture of fermented milk and yogurts, and therefore is more widely used by the industry. The ingredients (milk, whey, sugar, thickener, and stabilizer) are homogenized, heat-treated, and cooled, followed by the addition of lactic acid culture. Incubation is carried out at the optimum temperature of the lactic acid bacteria until reaching the desired acidity. Then, the product is subjected to cooling, addition of fruit preparations, flavorings and colorants, filling, and storage.

For the fermentation of whey beverages, the traditional cultures of *S. thermophilus* and *L. bulgaricus*, and more recently, probiotic cultures such as *L. acidophilus*, *L. casei*, *Bifidobacterium bifidum*, *Bifidobacterium longum*, *Bifidobacterium infantis*, and *Bifidobacterium adolescentis* have been used (Prudencio and Castro, 2011). The number of viable bacteria must be at least $10^6$ cfu/g throughout the product’s shelf life (Brasil, 2005).

In the second manufacturing process, whey basis is prepared by homogenization of whey and other ingredients (sugar, thickener, and stabilizer), followed by heat treatment and cooling to 20°C. Then, fermented milk, fruit preparations, flavorings, and colorings are added to the formulation, and subjected to packaging, cooling, and storage.
19.8.3 HEAT-TREATED FERMENTED WHEY BEVERAGES

The heat treatment of fermented whey beverages aims to increase their shelf life and allow storage at room temperature. Temperatures of 60–80°C are used after the fermentation process. Thus, the conventional fermented milk is stored under refrigeration (4°C) for up to 60 days, while heat-treated fermented whey beverages can be stored for several months at room temperature, which can reduce costs.

Therefore, this type of beverage does not contain viable bacteria; the fermentation process aims to reduce the lactose content and improve the sensory characteristics of the products.

19.8.4 NONFERMENTED WHEY BEVERAGES

Nonfermented whey beverages are mainly represented by the chocolate beverages. In addition, there are also those containing yogurt and fruit juice or shake-type beverages, with lower commercial representation in Brazil.

Chocolate whey beverages are primarily formulated with milk, sugar, and cocoa powder, as well as hydrocolloids that are used to improve consistency and prevent sedimentation of cacao particles. The
characteristics of each ingredient, such as milk fat, cocoa alkalinity, color powder, and stabilizer concentration can produce marked differences in composition and physical and sensory properties of the formulated products. The liquid nature of the chocolate whey beverages evidences the ingredients as cocoa flavor, which contributes to its great popularity.

There are three main types of chocolate whey beverages, classified according to the heat treatment: pasteurized, UHT, or sterilized. First, the dry ingredients are mixed to obtain a good particle rehydration, and improve powder dispersibility. Then, this mixture is homogenized with milk and other ingredients, and subjected to heat treatment according to the type of beverage, followed by cooling, packaging, and storage.

The packages are usually carton packs for UHT products, glass packaging for sterile products, and Tetra Top and Tetra Rex with carton body and plastic cover for pasteurized products. Pasteurized whey beverages can also be packaged in plastic bags.

The shelf life of the chocolate whey beverages depends on the type of heat treatment and packaging used. Although products in carton packaging are valid for 180 days, they must be consumed immediately (200 mL) or within 48 h (1 L) under refrigeration, after opening the packaging. Products sterilized in glass containers have shelf life of 180 days.

19.9 CONCLUSIONS

The Brazilian industry has expanded the range of fermented milk products, with improvements in sensory characteristics (appearance, aroma, flavor, and texture) aimed to increase consumption. Yogurts and fermented milk have higher nutritional value when compared to whey beverages, fermented or not. In turn, whey beverages have lower caloric value, lower cost, and confer health and environment benefits due to reutilization of whey. Thus, the most important factor in this context is to ensure proper labeling for consumers’ choice.

REFERENCES


DAHI—AN INDIAN NATURALLY FERMENTED YOGURT

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Anand Agricultural University, Anand, India

20.1 INTRODUCTION

Traditional foods in India are not only a part of the daily diet of the people of this subcontinent, but also a key to social and spiritual harmony. Traditional fermented foods have been prepared and consumed for thousands of years in India. Among them, fermented milk product, dahi, occupies a place of importance that the other traditional products cannot claim. It is believed that the Aryan-speaking people, who entered India between 1500 and 1000 BC, were responsible for bringing milk products such as dahi and ghee to India. The cow is considered a sacred animal in India, and cow’s milk and its products are used in every cultural and religious functions besides routine consumption. Dahi is consumed daily as part of regular diet. It is also used for the preparation of other culinary items. It is used as an ingredient of Panchamrita (a mixture of five foods used in Hindu worship and puja). Products based on dahi, namely, chhash, raita, and kadhi, are a daily fare in almost all households irrespective of the status of the people. Dahi is equivalent to yogurt in most aspects except those shown in Table 20.1. Products similar to dahi are known by different names throughout the world, namely, Laben in Egypt, Matzoon in Armenia, Gioddu in Italy, and Naga in Bulgaria. Dahi and its related products are significant to the Indian dairy industry because they act as an outlet for surplus dairy milk. These products have tremendous traditional appeal throughout the Indian masses and also abroad, especially the affluent consumer groups who have regional preferences in foods. The demand for fermented milk products is increasing and it has been estimated that about 10% of the total milk produced in India is used for the preparation of traditional fermented milk products, which has led to the increased large-scale commercial production of dahi, lassi, shrikhand, buttermilk, and misti doi. Dahi is considered a very good carrier of probiotics and a lot of research work has been carried out related to potential therapeutic aspects of dahi containing probiotic bacteria. This chapter deals with the various aspects, such as history, production, microbiology, nutritional, and therapeutic aspects, of the Indian naturally fermented food, dahi.

20.2 HISTORY

Dahi, called Dadhi in Sanskrit, has been mentioned in ancient Indian scriptures such as Vedas and Upanishads, and various hymns for which exact date is not known, but have been referred in about 10,000 years old era of Lord Rama. During Lord Krishna’s time (c.3000 BC), dahi, buttermilk and country butter were highly regarded. It is said that the Aryans consumed dahi in their daily diet because of its acidic, refreshing taste and
ability to reduce putrefactive changes. The traditional scientific system of Indian medicine, Ayurveda, in its well-recognized treatise, Charaka Samhita and Sushruta Samhita, discusses various properties of cow and buffalo milk dahi and emphasizes its therapeutic characteristics (Prajapati and Nair, 2008).

20.3 PATTERN OF CONSUMPTION AND USES
Dahi is consumed directly either in sweetened or in salted and spiced form. It is also consumed with other foods such as rice and chapatti. It is consumed more in summer than in winter. It is used for the preparation of products such as chaach, lassi, makhan, kadhi, and shrikhand. Dahi has found many nonfood applications in the day-to-day life of people in India. Idols of Gods made from metals are usually bathed in dahi first, followed by other items before routine worship in temples. In the traditional beauty regime of Indian women, dahi is considered as an enhancer of natural good looks and has been used for routine cosmetic applications. The lactic acid and enzymes in dahi help to hydrate and soothe the skin leaving it soft and smooth. The cleansing properties of dahi purify the skin as well when applied directly to the skin. As a sunburn remedy it soothes the pain and improves redness. Applied to the scalp it can relieve itchiness and reduce dandruff. When combined with honey and lemon juice, dahi makes a moisturizing and exfoliating mask, ideal for softening the face and neck and minimizing discoloration. Curd is a good hair conditioner. It provides good nourishment and adds a healthier look to hair (Prajapati and Sreeja, 2013).

20.4 METHODS OF PREPARATION OF DAHI
The traditional method for the preparation of dahi is typically a small-scale art or technology. It is prepared either in consumers’ households or in sweet makers’ shops in urban areas. In the household, milk is boiled, cooled to ambient temperature, and inoculated with a spoonful of (roughly 0.5%–2.0%)
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20.4 METHODS OF PREPARATION OF DAHI

starter (previous day’s dahi or buttermilk) and allowed to set overnight. It is then stored in a refrigerator and consumed. Small shopkeepers selling sweetmeats called Halwais make dahi by a short set method (curdling within 4–6 h). They use inoculum at the rate of 2%–4% followed by incubation at 42–45°C until setting of the curd. The inoculum is essentially the previous day’s dahi, which consists of mixed lactic acid bacteria. In cooler weather, the dahi setting vessel is usually wrapped in a woolen cloth to maintain warmth. In shops, the method is more or less the same except that milk is concentrated somewhat before inoculation and the dahi is usually set in a shallow circular earthen pot, which helps in the absorption of any whey that may ooze out.

Many organized dairies are now preparing dahi by adopting a standardized method. In this method, fresh, good quality milk is preheated and subjected to filtration and clarification, standardized to the desired fat and solid-not-fat level, homogenized, and heat treated followed by cooling to incubation temperature and inoculated with specific dahi starter culture. It is then poured into suitable containers (plastic cups) of the appropriate size and incubated. When a firm curd is formed and the acidity reaches about 0.7% lactic acid, dahi cups are transferred to a cold room maintained at about 4–5°C and stored at that temperature until consumption (Fig. 20.1). Dahi manufactured by commercial dairy plants is packaged in low-density polyethylene (LDPE) cups and heat sealed with aluminum foil. The marketing of dahi, especially in rural areas, is still carried out either through local vendors who travel by bicycle from one home to another or through dughdalayas (small shops where milk and milk products are

![FIGURE 20.1](image)

Commercial production of dahi (short set method).
produced and marketed). Big dairy plants market dahi through their market outlets meant for milk and milk products.

### 20.4.1 MILK QUALITY, ADDITIVES, AND PRETREATMENTS

The quality of dahi is largely defined by the composition and quality of milk used for its making because these parameters affect the starter activity as well as sensory characteristics of the dahi. The milk used for dahi making should be fresh, clean, and free from developed acidity, off-flavors, antibiotic residues, and inhibitory substances, which can adversely affect the starter activity. The chemical composition, status of fat, protein, minerals, enzymes, vitamins, etc. of dahi varies with the type of milk used in its manufacture. Milk of different species of mammals, namely, cow, buffalo, sheep, and goat, have been used for the production of dahi depending on availability (Table 20.2). “Yak” and “zomo” milk are used for the preparation of dahi in the Himalayas (Prajapati and Nair, 2008). The composition of milk has a significant effect on the flavor, appearance, body, textural characteristics, and pH of dahi. Dahi made from buffalo milk was reported to be firmer than the product made using cow’s milk or a mixture of cow’s and buffalo milk. Dahi made from goat’s milk displayed a firmer curd than cow’s milk product. The higher solids content of buffalo and goat’s milk contributes to body and textural characteristics of dahi. The level of total solids in milk has a significant effect on the firmness of dahi. As the total solids level increases, the curd gel firmness increases. The rate of acid production is faster in buffalo milk than in cow’s milk. Dahi made from concentrated milk showed no syneresis at refrigeration temperature (Reddy et al., 1987). Cow milk dahi shows better flavor because of high levels of volatile fatty acids, diacetyl, and acetoin compared to buffalo milk dahi. Milk with abnormal composition such as colostrum, late lactation milk, and mastitic milk should not be used because it adversely affects the growth of starter culture, which subsequently results in a poor quality product. Though colostrum is very unstable to heating, manufacturing of good quality curd from colostrum has been reported. Mastitis milk has an altered chemical, physical, and microbial profile because of reduction in synthesis, chemotaxis, and increased blood level in milk. These qualitative and quantitative alterations hinder the growth of lactic acid bacteria causing slow acid production. Higher somatic cell count in the milk reduces the rate of acid and diacetyl production in dahi (Saraswat and Agrawal, 1982). Singh and Singh

<table>
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<tr>
<th>Type of Milk</th>
<th>References</th>
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<tbody>
<tr>
<td>Buffalo milk and cow milk</td>
<td>Saraswat and Agrawal (1982) and Reddy et al. (1987)</td>
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<tr>
<td>Crossbred cow milk</td>
<td>Kulkarni et al. (1980) and Sanyal and Yadav (1986)</td>
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<tr>
<td>Goat milk</td>
<td>Singh et al. (1991) and Dorai et al. (2009)</td>
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<tr>
<td>Ewe milk</td>
<td>Rao and Basu (1952)</td>
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<tr>
<td>Vacuum-evaporated buffalo skim milk</td>
<td>Patel and Chakraborty (1985)</td>
</tr>
<tr>
<td>Yak milk</td>
<td>Tamang et al. (2007)</td>
</tr>
<tr>
<td>Reconstituted, toned, double-toned, recombined,</td>
<td>Kohk et al. (1980)</td>
</tr>
<tr>
<td>and standardized milk</td>
<td></td>
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<tr>
<td>UHT milk</td>
<td>Sharma and Prasad (1990)</td>
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<tr>
<td>Calcium-enriched buffalo milk</td>
<td>Singh et al. (2005) and Ranjan et al. (2006)</td>
</tr>
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have reported lower acidity, poor body and texture, and unsatisfactory flavor in buffalo milk dahi because of reduction in lactose (40%), decrease in αs1- and β-caseins, increase in γ-casein, and alkaline pH in high somatic cell count milk. Such dahi is considered to be a potential health hazard because of the presence of pathogens. The presence of antibiotic residue, detergents, and sanitizer residues has a profound inhibitory effect on the growth of the starter. Studies conducted on calcium fortification of cow and buffalo milk for dahi making revealed that among the three salts studied, namely, calcium chloride, calcium lactate, and calcium gluconate, the quality of dahi made from cow milk enriched with calcium gluconate (Singh et al., 2005) and buffalo milk enriched with calcium gluconate and calcium lactate (Ranjan et al., 2006) were comparable with the dahi made from the noncalcium fortified milk.

Pretreatments such as filtration, clarification, and bactofugation make the milk clean and bacteriologically better because these treatments help in the removal of dirt particles, bacterial cells, somatic cells, bacterial spores, etc. During the formation of dahi, butterfat has a tendency to rise to the surface. Garg (1988) reported that during fermentation, electrical charges on fat globules are neutralized causing the globules to coalesce and rise to the top. To prevent this, homogenization is a desirable additional step in the manufacture of dahi. Homogenization splits fat into smaller globules and prevents fat separation during incubation. Use of homogenization for milk meant for dahi increases the viscosity and coagulum stability and also reduces whey separation of dahi during storage. It also improves the mouthfeel of the product and thus increases organoleptic quality (Tamime and Deeth, 1980). The effect of homogenization on whey proteins has been reported by Dave (1984). Relatively higher temperature and pressure employed during homogenization causes denaturation of serum proteins, thus improving the hydrophilic property of the gel. In spite of these advantages, homogenization of milk is not followed by the unorganized sector. Homogenization is said also to have certain disadvantages, e.g., the mechanical force applied for breaking up of fat globules during homogenization can result in the breakup of bacterial clumps leading to an increase in total plate count. Also the homogenizer can be an additional source of contamination if not cleaned and sanitized properly.

Various additives are frequently used to control the texture and also reduce whey separation in dahi. However, sugar is the only legally permitted additive in dahi. Incorporation of additives such as starch, alginate, and gelatin improve the quality of dahi (Jogand et al., 1991). Different salts and chemical additives have varied effects on the quality of dahi. Calcium chloride and magnesium chloride have a greater effect on reduction of curd tension than sodium salts. The addition of 0.2% citrate is recommended to enhance the pleasing aroma of dahi (Prajapati and Sreeja, 2013).

### 20.4.2 HEAT TREATMENT OF MILK

Traditionally, boiled milk is used for dahi preparation. Industrial production of dahi makes use of higher temperatures (85°C/30 min or 95°C/10 min) than normal pasteurization. Such high heat treatment improves milk as a medium for the growth of starters and gives many other benefits, which are mentioned in Table 20.3. Different heat treatments exert varied effects on the final quality of dahi. A satisfactory product quality is obtained when a heat treatment of 85°C for 30 min is used for heating milk. Boiling improves culture activity causing faster coagulation (Kohk et al., 1980; Saraswat and Agrawal, 1982). Lactococcus lactis C10 and Streptococcus thermophilus produce dahi with maximum acidity when milk is heated at 63°C for 30 min, while Lactococcus cremoris C1, Lactococcus diacetylactis DRC1, and Lactobacillus delbrueckii ssp. bulgaricus produce maximum titratable acidity in milk.
heated at 85°C for 10 min. Kohk et al. (1980) found that heat treatment of milk at 71.5°C for 15 s had no significant effect on the titratable acidity of resultant dahi. On the basis of objective and subjective evaluation, Garg and Jain (1980) found that heating of milk at 82.2°C for 16–20 s was best for dahi making. The relative viscosity of dahi from boiled milk was more compared to pasteurized milk and the relative viscosity of dahi from recombined milk was observed to be more in both heat processing methods (Kohk et al., 1980). Boiling increased curd tension in dahi compared to pasteurization. The microstructure of dahi is influenced by the level of heating applied to the milk. According to Tomar and Prasad (1989) buffalo milk heated to 70°C resulted in a product that was soft, had an open structure, and the casein was near spherical in shape (i.e., a size of about 300 nm), while milk heated at 90°C for 30 min gave a firm curd and the micelle size was about 235 nm and elongated in shape; the protein matrix consisted of a long micellar chain. Kohk et al. (1980) reported a higher diacetyl production in dahi from boiled milk.

### 20.4.3 STARTER CULTURES

Different methods for the curdling of milk are described in different texts for the preparation of dahi. The method described in Taittiriya Samhita mentions the use of whey and rice grains for the curdling of milk (Roy, 1997). The starter culture used for dahi making is of utmost importance because of the inevitable role (Table 20.4) it plays during the entire manufacturing process. To manufacture dahi on a large scale with predictable uniform quality, it is desirable to use known mixtures of starters. Usually, the starter bacteria consist of *L. lactis* ssp. *lactis*, *L. lactis* ssp. *cremoris*, *L. lactis* ssp. *diacetylactis*, *Leuconostoc* spp., *Lactobacillus* spp., and *S. thermophilus* (Fig. 20.2A and B). Lactobacilli dominate in sour dahi because of their higher acid resistance, while streptococci dominate in sweet dahi. Surveys

<table>
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<th>Table 20.3 Advantages of High Heat Treatment</th>
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<tbody>
<tr>
<td>1. Preparation of a relatively sterile medium for the exclusive growth of starter culture</td>
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<tr>
<td>2. Inactivation of natural inhibitory substances, enzymes, etc. present in milk</td>
</tr>
<tr>
<td>3. Expulsion of air from the medium to produce a more conducive medium for the growth of microaerophilic lactic cultures</td>
</tr>
<tr>
<td>4. Thermal breakdown of milk constituents, mainly proteins and thus the release of peptones and sulfhydryl groups, which provide nutrition and anaerobic conditions for the starter growth</td>
</tr>
<tr>
<td>5. Denaturation and coagulation of milk albumins and globulins enhance the viscosity and help in producing a firm consistency in the dahi</td>
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<tr>
<th>Table 20.4 Role of Starter Culture in Dahi Making</th>
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<tbody>
<tr>
<td>1. Production of lactic acid</td>
</tr>
<tr>
<td>2. Coagulation of milk and therefore changes in the body and texture of the product</td>
</tr>
<tr>
<td>3. Production of volatile flavor compounds such as diacetyl, acetaldehyde, etc.</td>
</tr>
<tr>
<td>4. Controlled proteolytic and lipolytic activity</td>
</tr>
<tr>
<td>5. Production of other compounds such as vitamins, other acids, CO₂</td>
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<tr>
<td>6. Production of antibacterial substances</td>
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<td>7. May add health properties to the product</td>
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</tbody>
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FIGURE 20.2
(A) Lactococci/streptococci and (B) lactobacilli.

Courtesy: SMC College of Dairy Science, Anand.

FIGURE 20.3
Freeze-dried dahi starter cultures in vials, ampoules, and pouches.

Courtesy: SMC College of Dairy Science, Anand.
have also indicated that in summer, dahi lactobacilli dominate, while in winter, streptococci dominate. Similarly, in southern parts of India, where people are habituated to take sour dahi, lactobacilli and yeast dominate, while in northern parts where mildly sour dahi is preferred, streptococci predominate (Prajapati, 1995). The starter cultures for dahi making are commercially available in the form of liquid, concentrated, and freeze-dried cultures (Fig. 20.3) from culture collection centers and institutes. Alternatively, a direct vat inoculum/direct vat starter concentrated culture can be used directly in a fermentation vat for the preparation of dahi.

20.4.4 INOCULATION, PACKAGING, AND INCUBATION

The amount of inoculum used for the production of cultured milk products depends on the type of culture and incubation period desired. The use of a minimum amount of culture causes greater fluctuations in acid production and a high amount will result in defects in the texture and aroma of the final product. An optimum amount of inoculum will result in the normal course of lactic acid fermentation. The inoculated milk is either retail packed or set in bulk. The packaging before or after setting depends upon whether the product is a stirred type or a set type. The incubation period is generally guided by the rate of cooling and final acidity desired in the product.

Dahi manufactured by commercial dairy plants is usually packaged in LDPE cups and heat sealed with aluminum foil. The other commonly used plastics for the packaging of dahi are polypropylene, polyvinylchloride, and polyvinylidene chloride (Goyal, 1986). Cup filling and sealing machines are suitable for filling and sealing dahi in preformed cups. These may be of rotary or straight through type. Dahi is available in pouches too. Some dairy plants fill the inoculated milk in pouches and transfer them to the incubation room for setting the dahi; these are then marketed. Form-fill-seal machines are widely used for packaging in pouches.

Traditionally, earthen pots or kulhars are used for setting as well as selling large quantities of dahi. Dahi packaged in kulhars has a firm body and these packages are eco-friendly and easily degradable after disposal; however, kulhars are heavy, breakable, and can cause excessive shrinkage of the product during storage because of moisture seepage through pores. There are also problems of maintaining high standards of hygiene and proper sealing. Dahi in mud pots shows heavy contamination with yeast and mold, but 90%–99% of contaminants can be killed by submerging the pots in boiling water for 1 min. Kulhars coated with shellac were tried for the packaging of dahi. Such kulhars significantly reduced the moisture loss and microbial load from dahi during storage. Dried dhak leaf cups are also traditionally used for packaging dahi (Paltani and Goyal, 2007).

20.4.5 COOLING

The product is immediately cooled to less than 5°C after setting by exposing cups to high-velocity air in the cold room to arrest excessive acid development and “whey-off” (Aneja et al., 2002). Cooling is essential to arrest fermentation. Hence, keeping in mind the acidity that will develop during the process of cooling, the incubation step should be terminated. Rate of cooling has a great effect on the body and textural characteristics of dahi. Generally, faster cooling results in contraction of gel leading to more whey separation. A slow rate of cooling allows time for adjustment in the gel and helps to retain more water. Usually for dahi incubated at higher temperature, a two-stage cooling is recommended, i.e., from
about 40°C to 20°C in the first stage and from 20°C to 5°C in the second stage, usually in the cold store. Cooling should cause minimum mechanical damage to the curd, because the coagulum is in the most sensitive stage (Prajapati and Sreeja, 2013).

### 20.5 MICROFLORA OF DAHI

In addition to several species of lactic acid bacteria (Dave et al., 1991; Sharma et al., 1993; Prajapati and Sreeja, 2013), lactose fermenting yeasts (Shilpa and Gandhi, 1993), coliforms (Jayaram and Gandhi, 1987), spore formers (Jayaram and Gandhi, 1987), spores and staphylococci have been reported as microflora in dahi. Yeasts and molds appear to be the most common contaminants in dahi (Sharma et al., 1993). These organisms enter the product from the atmosphere, utensils, and human hands under natural conditions. Because of their ability to tolerate acidic environments, yeasts can multiply in dahi producing gassiness and flavor defects associated with lipolysis of milk fat, if the product is held too long at ambient temperatures. Although the conditions present in dahi are not favorable for the growth of contaminants, different pathogenic organisms have been found to survive in dahi. The organisms, because of their variable degree of survival, namely, *Staphylococcus aureus* (Kahlon and Grover, 1984), *Escherichia coli* (Ghosh and Rajorhia, 1987), *Enterobacter aerogenes* (Prasad et al., 1980), and *Salmonella paratyphi*, can be a potential threat.

### 20.6 DAHI IN HEALTH AND DISEASE PREVENTION

The Ayurvedic system of medicine in India recognized the value of dahi and buttermilk as therapeutic agents in the treatment of gastrointestinal disorders and other human ailments quite a long time ago. *Charaka Samhita* in 1000 BC mentions *jatharagni* (fire in the stomach) as the sustaining force of all living beings and states that the first line of treatment for disease related to the stomach is *Takra*, i.e., a fermented milk called *chhash*. Ayurveda also describes the properties of various types of *chhash* and their role in the control of intestinal disorders. There is a mention of *karambha* (blended curd–rice dish) in *Rigveda* (Prajapati and Nair, 2008). The beneficial effect of fermented milk in the treatment of intestinal diseases may be ascribed partly to the effect of lactic acid and antibacterial substances present in them and partly to the creation of favorable conditions for the growth of *Lactobacillus acidophilus*, which is a normal inhabitant in the intestine and plays a key role in controlling the microbial population there. Also the consumption of fermented milk has the potential to increase gut bacterial colonization via the protective effect of milk protein for survival through gastrointestinal transit (Fernandez et al., 2003). A lot of research work is being done on the therapeutic aspects of dahi.

#### 20.6.1 NUTRITIVE ASPECTS

Nutritive value of dahi is mainly dependent on the type of milk used for its production, the process conditions and the types of cultures used. Dahi contains all the valuable constituents of milk except for the changes caused as a result of heat treatment of milk, microbial growth, and fermentation processes. The residual amount of lactose after its partial utilization by lactic acid bacteria for
production of lactic acid and other compounds contributes to the calorific value of dahi and may help in alleviating the adverse effects of lactose intolerance (Ayebo and Shahani, 1980). The native milk proteins are converted into a soft curd containing finely dispersed casein particles caused by bacterial action in the fermented product. As a result, fermented milk proteins are more easily digested and assimilated than milk proteins and are therefore particularly useful to children, old people, and persons suffering from stomach ulcers who cannot tolerate milk proteins in their original form. Dahi is said to have 3%–30% higher net protein quality than the milk from which it is prepared. The net protein utilization, protein efficiency ratio, biological value, and percent digestibility of dahi are reported to be 82±0.25, 3.2, 92±0.05, and 93.8±1.15, respectively (Sarkar, 2008). Studies conducted at the University of Agricultural Sciences, Bangalore, have indicated that the essential amino acid contents of microbial cell proteins in dahi prepared with different starter cultures ranged from 2.0 to 6.5 mg per 100 g of dahi. This may appear to be negligible from a quantitative point of view compared to the total essential amino acid content of dahi protein (1470–2433 mg per 100 g). From a qualitative standpoint, however, the essential amino acid contents of microbial protein become significant since the cells of some bacteria are rich in methionine, lysine, and cysteine contents, which would be helpful in correcting any marginal deficiencies in the diet with respect to these amino acids. Similarly, the free amino acids released in the medium by different cultures included appreciable amounts of methionine, lysine, and cysteine (Laxminarayana, 1984). By using selected cultures of lactic acid bacteria in a suitable combination it may be possible to enhance the vitamin contents as well as protein quality of dahi to a considerable extent without affecting the desirable flavor characteristics of the product. The incorporation of a culture of Propionibacterium shermanii in the starter was particularly useful in increasing the vitamin B12 content of the product. Production of free fatty acids and conjugated linoleic acid in probiotic dahi containing L. acidophilus and L. casei during fermentation and storage was reported by Yadav et al. (2007a). No change was observed in the control dahi. Probiotic lactobacilli appeared to increase the production of free fatty acids by lipolysis of milk fat and produced conjugated linoleic acid by using internal linoleic acid, which may confer nutritional and therapeutic value to the product.

### 20.6.2 Dahi and Diarrheal Diseases

Dahi and buttermilk have been used as a reliable household remedy for diarrheal diseases since ancient times. Dahi was mentioned in the Ayurveda literature from around AD 600 for the treatment of diarrhea. In Charaka Samhita, Charaka advocates the use of food preparations using curd for diarrheal diseases. Buttermilk (Takra) mixed with cumin, asafoetida, and rock salt is said to cure diarrhea. Such a recipe can provide a good amount of protein, minerals, sugar, vitamins, and water, which are essential for overcoming the malnourishment and dehydration caused by diarrhea. In India, curd with khichari is the most preferred food item for the management of these diseases. Because of its appetizer, anti-diarrheal, and easily digestible properties, buttermilk is also recommended for gastrointestinal disturbances other than diarrhea such as ascites and hemorrhoids. Sour curd is preferred in the Ayurvedic system for diarrheal disorders compared to the sweet version. Dahi can significantly reduce the duration of diarrhea in children (Agarwal and Bhasin, 2002). Indian physicians used fat-free curd/buttermilk orally as well as in enemas to treat chronic gastrointestinal disorders such as colitis and chronic diarrhea. Dahi along with a usual diet is known to reduce the number of episodes as well as duration of diarrhea. Dahi is also believed to improve appetite and vitality.
20.6.3 ANTIMICROBIAL PROPERTIES OF DAHI

Along with the lower pH, the antimicrobial compounds produced by the fermenting flora in dahi prevent the growth of undesirable organisms. The effect of acidity is relatively small compared to specific substances of an antibiotic nature (bacteriocins) produced by the fermenting organisms. Many lactobacilli exhibited antibacterial action against pathogenic strains of staphylococci and E. coli and the antibacterial action was a function of pH because the culture lost antibacterial activity when pH was raised to 5.0 or above. Various degrees of inhibition were shown by dahi against E. aerogenes, S. aureus, E. coli, Bacillus subtilis, S. paratyphi, Salmonella typhimurium, and Campylobacter jejuni. Dahi prepared using S. thermophilus strains exhibited antibacterial activity against E. coli, Bacillus cereus, S. aureus, Salmonella typhosa, and Pseudomonas aeruginosa (Prajapati and Sreeja, 2013). Dave et al. (1992) determined the antibacterial activity of dahi against B. cereus, S. typhosa, E. coli, and S. aureus when dahi was prepared using the MD8 strain of S. thermophilus. In another study, Balasubramanyam and Varadaraj (1994) reported antibacterial activity against B. cereus and Bacillus licheniformis. Dahi prepared using a nisin-producing strain L. lactis W8 displayed antibacterial property against spoilage and pathogenic bacteria including Listeria monocytogenes. When L. monocytogenes was mixed with dahi at 5.2 log cfu/mL and stored at 4°C, the number of L. monocytogenes gradually decreased and became undetectable at 10 h (Mitra et al., 2010).

20.6.4 IMMUNE ENHANCEMENT

A study on the augmentation of immune response in mice fed with dahi made using Leuconostoc citrovorum and L. lactis showed that dahi activates the nonspecific immune system and also protects against enteric infection by Shigella dysenteriae (Singh and Kansal, 2003). Jain et al. (2009) evaluated the effect of dahi containing probiotic L. casei to modulate immune response against Salmonella enteritidis infection in mice and found that probiotic dahi was more efficacious in protecting against S. enteritidis infection by enhancing innate and adaptive immunity than fermented milk and normal dahi. Hence, dahi may strengthen the consumer’s immune system and may protect against infectious agents such as S. enteritidis. Rajpal and Kansal (2009a,b,c) studied the protective effect of buffalo milk probiotic dahi containing selected strains of L. acidophilus and Bifidobacterium bifidum and using dahi culture (L. lactis ssp. cremoris and L. lactis ssp. lactis biovar. diacetylactis) on intestinal infection in mice fed with the product for 2–7 days and then challenged with S. dysenteriae. The colonization of Shigella in the intestine, liver, and spleen was significantly lower in mice fed on probiotic dahi. Dewan et al. (2009) studied the cytokine response to dietary rehabilitation with dahi and leaf protein concentrate in malnourished children. Protein energy malnutrition, an important cause of secondary immune deficiency, is associated with several abnormalities in the immune system including cytokine production. In this study cytokine levels, both pro- and antiinflammatory, were evaluated in protein energy malnourished children following nutritional rehabilitation with dahi and leaf protein concentrate (LPC). They suggested that cytokines (tumor necrosis factor alpha, interferon gamma, interleukin (IL)-10, and IL-4) may serve as biological markers to assess the effect of functional foods such as curd or LPC on immunity in malnutrition. Curd may help to maintain the balance in cytokine production by increasing the production of IL-10 and may be considered in place of milk in the nutritional rehabilitation of malnourished children.
CHAPTER 20 DAHI—AN INDIAN NATURALLY FERMENTED YOGURT

20.6.5 LACTOSE INTOLERANCE

Lactose intolerance is a physiological state in human beings where they lack the ability to produce an enzyme called lactase or β-galactosidase. Lactase is essential to breakdown lactose in milk into glucose and galactose. Individuals lacking lactase will not be able to digest milk and it often poses a problem in newborn infants. People with lactose intolerance express abdominal discomfort, diarrhea, cramps, flatulence, nausea, vomiting, etc. Another problem associated with lactose intolerance is calcium deficiency. The residual amount of lactose after its partial utilization by lactic acid bacteria for the production of lactic acid and other compounds may help in alleviating the adverse effects of lactose intolerance (Ayebo and Shahani, 1980).

20.6.6 BLOOD PRESSURE REDUCTION/CHOLESTEROL REDUCTION/ANTIATHEROGENIC EFFECT

An acidophilus dahi containing live lactobacilli count of 200 million cfu/g at the end of its shelf-life had been validated by human feeding trials for its ability to reduce cholesterol. The study resulted in average significant reduction in the serum total and low-density lipoprotein (LDL) cholesterol. In the majority of cases, a significant change in lipid profile parameters was observed after 10 and 20 days of feeding, indicating a gradual effect. A placebo-controlled study to test the effect of dahi containing L. delbrueckii ssp. bulgaricus, S. thermophilus, and L. lactis biovar. diacetylactis on 29 hypertensive subjects in three age groups (35–45 years old, 45–55 years old, and 55–65 years old) showed that the serum cholesterol of all 19 participants who consumed dahi was reduced significantly (Ashar and Chand, 2004). The cholesterol-lowering property of probiotic dahi containing selected strains of L. acidophilus and B. bifidum was evaluated in rats by Rajpal and Kansal (2009a,b,c). They found that the cholesterol and plasma triacylglycerol (TAG) contents in the liver were lower in the probiotic dahi group than in the control group. Probiotic dahi decreased diet-induced hypercholesterolemia and reduced atherogenic index by increasing high-density lipoprotein (HDL) and attenuating the rise in TAG in a hypercholesterolemic diet.

Sinha and Yadav (2007) studied the antiatherogenic effect of probiotic dahi prepared using a culture with probiotic L. acidophilus and L. casei in rats fed with a cholesterol-enriched diet. They found that plasma total cholesterol was suppressed to the extent of 22%–28% in hyperlipidemic rats fed lyophilized probiotic dahi as well as lyophilized cells of broth probiotic cultures. Plasma triglycerides also decreased significantly (P < .05). There was 41%–56% suppression in LDL-cholesterol in probiotic dahi and culture-fed groups but HDL-cholesterol was not affected. The atherogenic index was reduced significantly in probiotic-fed groups when compared with the reference diet-fed group.

20.6.7 ANTIDIABETIC EFFECT

The antidiabetic effect of dahi containing L. lactis was observed on high fructose-induced diabetic rats. The fasting blood glucose, glycosylated hemoglobin, insulin, free fatty acids, and triglyceride levels of the dahi-fed group animals were significantly lower than those of the control group (Yadav et al., 2006). The work done by Yadav et al. (2008a) suggests that the supplementation of probiotic L. acidophilus and L. casei with dahi cultures increased the efficacy of dahi to suppress streptozotocin-induced diabetes in rats by inhibiting depletion of insulin as well as preserving diabetic dyslipidemia and inhibiting lipid peroxidation and nitrite formation. This may empower the antioxidant system of β-cells and may slow down the reduction of insulin and elevation of blood glucose levels. They also
reported that the oral administration of probiotic dahi significantly increased counts of lactobacilli adherent to epithelial walls and free in the lumen of the small and large intestine, while decreasing attached as well as free coliform counts (Yadav et al., 2008b).

### 20.6.8 ANTICARCINOGENIC EFFECT

Rajpal and Kansal (2008) demonstrated a reduction of gastrointestinal cancer in rats fed with buffalo milk probiotic dahi containing selected strains of *L. acidophilus* and *B. bifidum* and using dahi culture (*L. lactis* ssp. *cremoris* and *L. lactis* ssp. *lactis* biovar. *diacetylactis*). Probiotic dahi decreased lipid peroxidation and β-glucuronidase activity in the gut and increased glutathione-S-transferase, a carcinogen detoxifying enzyme activity in liver, which resulted in the attenuation of carcinogenesis in the gastrointestinal tract.

### 20.6.9 ANTIOXIDATIVE EFFECT

Probiotic dahi prepared by coculturing selected strains of *L. acidophilus* and *B. bifidum* and dahi culture in buffalo milk was evaluated for its antioxidative effects in rats. The activities of superoxide dismutase (SOD) and catalase in red blood cells increased in the case of probiotic dahi. In the liver, SOD activity was stimulated by probiotic dahi only, while in colorectal tissue both normal and probiotic dahi effectively stimulated SOD as well as catalase activity and thus improved antioxidant status in rats. Probiotic dahi was more efficacious than control dahi (Rajpal and Kansal, 2009a,b,c).

### 20.6.10 ANTIALLERGIC EFFECT

Jain et al. (2010) observed that feeding of probiotic dahi is remarkably effective in suppressing ovalbumin-induced allergic consequences in mice. Ovalbumin-stimulated lymphocyte proliferation was strongly suppressed by feeding probiotic dahi in comparison to milk and control dahi. The increased biological efficacy of probiotic dahi compared to control dahi might be caused by the higher content of bioconstituents such as conjugated linolenic acid (CLA), free fatty acid, complex oligosaccharides, and bioactive peptides in probiotic dahi. Probiotic dahi is said to contain a higher amount of CLA, free fatty acids (Yadav et al., 2007a), oligosaccharides (Yadav et al., 2007b) (prebiotics), and bioactive peptides (Jain et al., 2009), which are well known for their immuno-modulatory functions (O’shea et al., 2004; Stulnig, 2003; Metugriachuk et al., 2006; Silva and Malcata, 2005).

### 20.7 VALUE ADDITION TO DAHI

A number of ingredients and methods have been evaluated for value addition to dahi. One of the most important functional ingredients/organisms evaluated is probiotics (Yadav et al., 2005; Prajapati and Sreeja, 2013; Panjagari et al., 2016). Probiotic dahi is a regular curd with the added advantage of unique probiotic cultures such as *L. acidophilus* and/or *B. bifidum*. Dahi, which is already considered good for digestion, is the most appropriate product to introduce probiotics. The major commercial food manufacturers in the probiotic category in India are Amul, Nestlé, Mother Dairy, and Yakult India. Dairy Science colleges and
related educational institutions in India have carried out extensive research on value addition to dahi. At the Dairy Science College, Anand (Gujarat), India, an acidophilus dahi was developed that had a live lactobacilli count of 200 million cfu/g at the end of its shelf-life, which makes it therapeutically more important. This effect had also been validated by human feeding trials, which showed average significant reduction in the serum total and LDL-cholesterol. A synbiotic dahi was developed using the probiotic culture *Lactobacillus helveticus* MTCC 5463 and functional ingredient (prebiotic) inulin. The synbiotic dahi was stable during storage of 28 days at 4°C. Several other dahi-based synbiotic products such as synbiotic raita, synbiotic lassi, synbiotic whey drink, herbal probiotic lassi, and carbonated probiotic milk have also been developed. All the products were organoleptically acceptable up to 3–4 weeks at refrigeration temperature and had an optimum dose of probiotic lactobacilli (Prajapati and Sreeja, 2013). Behare et al. (2009) assessed exopolysaccharide-producing mesophilic strains to reduce whey separation and improve sensory aspects in dahi. A nisin-producing bacterial strain *L. lactis* W8 was used for extending the shelf-life of dahi (Mitra et al., 2010). Fortification with minerals for improving calcium content was evaluated by Singh et al. (2005) and Ranjan et al. (2006). Fruits are being incorporated into dahi to improve nutritional as well as therapeutic value through phytoneutrants and dietary fibers (Khurana and Kanawjia, 2007; Panjagari et al., 2016; Prajapati and Sreeja, 2013). Kale et al. (2011) conducted a study on the development of value-added dahi by incorporating fruit pulps, cereal flour, and whey proteins. Oat flour, whey protein concentrate, papaya, fig pulps, and sugar with different combinations were tried. The value-added dahi had a shelf-life of 7 days at 5°C. Value addition improved the firmness and flavor of the curd. Artificial sweeteners such as sac-sweet and sucrol were assessed for incorporation into dahi meant for diabetic patients (Islam et al., 2010). Enrichment of dahi with aloe gel as a functional ingredient was studied by Pushkala and Srividya (2011). Enrichment of dahi with aloe gel improved its quality characteristics by causing a significant reduction in whey syneresis and an increase in total solid content, water-holding capacity, total yield, and viscosity. In a study by Sivakumar et al. (2008), β-carotene-rich sweet potato curd was prepared by cofermenting 16% sweet potato with cow’s milk. They reported that addition of sweet potato enriched the curd with dietary fiber and starch improved the firmness of the curd and consumer acceptability. According to the researchers this low-cost and nutritious product has acceptable culinary quality that could help disadvantaged people to combat vitamin A deficiency in developing countries. Blending of sweet corn milk with buffalo milk for the preparation of low-cost nutritious dahi was reported by Padghan et al. (2015).

### 20.8 Conclusion

Dahi has been produced and consumed since ancient times in India and it has maintained its popularity in the Indian diet despite changing lifestyles and food habits over time. It is preferred over milk because of its good taste, high nutritive and therapeutic value, and, most importantly, enhanced keeping quality in a tropical climate. It is being consumed in different varieties such as plain, sweetened, salted, and spiced. At present, several types of dahi-based products such as mishti dahi, fruit dahi, probiotic dahi, lassi, shrikhand, and chhash are being produced and marketed. The therapeutic effects of dahi were recognized by the people of India long time ago, but the scientific validation of the same has happened only in the recent decades. As a result, dahi and its related products are gaining popularity as functional foods. The use of dahi-containing probiotics in intestinal therapy or in the promotion of general health of the population has not received sufficient attention in India. More clinical trials involving human volunteers are needed to prove the therapeutic aspects of dahi and its related products. However, dahi and its related products contribute significantly to the dairy business in India.
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PART

IMPORTANT MICRONUTRIENT AND BIOTIC COMPONENTS OF YOGURTS
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21.1 INTRODUCTION

As a nutritional food, milk is an important source of high-quality proteins, essential amino acids, and unsaturated fatty acids. Milk also contains a number of minor constituents including enzymes, vitamins, minerals, flavor compounds, and bioactive peptides. All of these constituents make important contributions to the nutritional and technological properties of milk and dairy products (Fox and McSweeney, 1998). Cow’s milk proteins contain approximately 80% of casein, consisting of alpha s1-, alpha s2-, beta-, and kappa-casein (αs1-CN, αs2-CN, β-CN, and κ-CN, respectively) (Kopf-Bolanz et al., 2012). Milk proteins play a central role in the formation of texture of cheese and yogurt. In addition, proteins provide a source of essential amino acids and bioactive peptides including angiotensin-converting enzyme (ACE)-inhibitory, antihypertensive, antibacterial, and opioid peptides (Donkor et al., 2007; Minervini et al., 2003; Muguerza et al., 2006; De Noni and Cattaneo, 2010). These peptides are encrypted in an inactive form in parent proteins and may be released by hydrolysis of bacteria-derived enzymes during fermentation of milk and storage of dairy products or digestive enzymes in the gastrointestinal tract (Choi et al., 2012; Sienkiewicz-Szlapka et al., 2009). Beta-caseins (β-CN)s derived opioid peptides were discovered for the first time during in vitro studies by Brantl et al. (1979) and were called beta-casomorphins (BCMs). These days, BCMs have been found in milk, cheese, and fermented milk (De Noni and Cattaneo, 2010; De Noni et al., 2015; Matar and Goulet, 1996; Nguyen et al., 2015a).

The identification of BCMs in dairy products, especially in yogurt, is a challenging task for chemical analysts. This is because yogurt is a complex food matrix containing a large number of proteins and peptides potentially interfering with BCMs. Furthermore, BCMs have been reported at rather low concentrations in yogurt (e.g., sub-ng/g–ng/g level), with BCMs also degrading during yogurt processing, which makes their analytical determination even more challenging (Nguyen, 2016).

This chapter focuses on reviewing the source and the occurrence of BCMs in yogurt and dairy products. The impact of processing conditions such as starter cultures, conditions of fermentation, and storage on the formation/degradation of BCMs during yogurt production is also discussed. In addition, state-of-the-art analytical techniques currently in use for identification and quantification of BCMs are critically discussed on the basis of the most recent scientific literature.
21.2 SOURCE OF BETA-CASOMORPHINS

BCM is a group of exogenous peptides with opioid property released from milk β-CN by enzymes during fermentation or in vivo or in vitro digestion (Brantl et al., 1979; Jinsmaa and Yoshikawa, 1999; De Noni, 2008; Boutrou et al., 2013). Most BCMs are derived from β-CN at positions 60–70 and contain the same first three amino acids (i.e., -Tyr-Pro-Phe-) in the sequence (Table 21.1) (Boutrou et al., 2013). Among these peptides, BCM5 and BCM7 representing fragments β-CNf60–64 and β-CNf60–66, respectively (Fig. 21.1), are the most important peptides with the strongest opioid property and currently studied more extensively for their biological activity (Kálmán et al., 1992; Brantl et al., 1981).

Results from in vitro studies have suggested that the release of BCM7 by hydrolysis of bovine β-CN depends on the presence of β-CN A1 and B variants. Both variants contain a histidine (His) residue at position 67 on sequence, while β-CN A2 variant contains a proline (Pro) residue at the same position, leading to prevention of cleavage of an isoleucine–proline (Ile–Pro) peptide bond (Fig. 21.2) (De Noni, 2008; Jinsmaa and Yoshikawa, 1999; De Noni et al., 2015). In human studies, the release of BCM7 from milk protein during in vivo digestion has been reported, but the presence of BCM7 and other BCMs in blood after the intake of milk or casein has not been demonstrated. More recently, BCM7 has been detected and quantified in a number of pasteurized milk products and the amount of peptide seems to depend on the amount of β-CN A1 variant in raw milk. Potential factors for the release of BCM7 in these dairy products are possibly native milk enzyme- or bacteria-derived enzyme action in raw milk.

<table>
<thead>
<tr>
<th>BCMs</th>
<th>Sequences</th>
<th>References</th>
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<tbody>
<tr>
<td>Beta-casomorphin 3, β-CNf(60–62)</td>
<td>Tyr-Pro-Phe</td>
<td>De Noni et al. (2015)</td>
</tr>
<tr>
<td>Beta-casomorphin 4, β-CNf(60–63)</td>
<td>Tyr-Pro-Phe-Pro</td>
<td>Kamiński et al. (2007), Matar and Goulet (1996), Brantl et al. (1981), and De Noni et al. (2015)</td>
</tr>
<tr>
<td>Beta-casomorphin 5, β-CNf(60–64)</td>
<td>Tyr-Pro-Phe-Pro-Gly</td>
<td>Kamiński et al. (2007), Juan-García et al. (2009), De Noni (2008), Brantl et al. (1981), and Nguyen et al. (2014)</td>
</tr>
<tr>
<td>Beta-casomorphin 6, β-CNf(60–65)</td>
<td>Tyr-Pro-Phe-Pro-Gly-Pro</td>
<td>Kamiński et al. (2007) and De Noni et al. (2015)</td>
</tr>
<tr>
<td>Beta-casomorphin 7, β-CNf(60–66)</td>
<td>Tyr-Pro-Phe-Pro-Gly-Pro-Ile</td>
<td>De Noni (2008), Kamiński et al. (2007), Juan-García et al. (2009), Brantl et al. (1981), and Nguyen et al. (2014, 2015a)</td>
</tr>
<tr>
<td>Beta-casomorphin 8, β-CNf(60–67)</td>
<td>Tyr-Pro-Phe-Pro-Gly-Pro-Ile-Pro</td>
<td>Kamiński et al. (2007)</td>
</tr>
<tr>
<td>Beta-casomorphin 9, β-CNf(60–68)</td>
<td>Tyr-Pro-Phe-Pro-Gly-Pro-Ile-Pro-Asn</td>
<td>Saito et al. (2000) and Jinsmaa and Yoshikawa (1999)</td>
</tr>
<tr>
<td>Beta-casomorphin 10, β-CNf(60–69)</td>
<td>Tyr-Pro-Phe-Pro-Gly-Pro-Ile-His-Asn-Ser</td>
<td>Toelstede and Hofmann (2008)</td>
</tr>
<tr>
<td>Beta-casomorphin 11, β-CNf(60–70)</td>
<td>Tyr-Pro-Phe-Pro-Gly-Pro-Ile-Pro-Asn-Ser-Leu</td>
<td>Kamiński et al. (2007)</td>
</tr>
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rather than the pasteurization process itself. However, to date, specific enzymes contributing to the formation of BCM7 in dairy products are still unknown (Nguyen et al., 2015a).

Epidemiological studies have reported that the consumption of milk containing β-CN A1, which releases BCM7, is linked to an increased risk of type 1 diabetes and heart disease (Birgisdottir et al.,...
2006; Elliott et al., 1999; McLachlan, 2001). However, the European Food Safety Association concluded that there is insufficient data demonstrating the relationship between BCM7 and other related BCMs and noncommunicable diseases (EFSA, 2009). Nonetheless, BCM5 and BCM7 have been linked to autistic children and apnea in infants (Sokolov et al., 2014; Wasilewska et al., 2011; Sun et al., 2003).

### 21.3 OCCURRENCE OF BETA-CASOMORPHINS IN YOGURT

#### 21.3.1 PRODUCTION OF YOGURT

Yogurt is a popular dairy product mainly produced from bovine milk by fermentation with two lactic acid bacteria (LAB), *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus* (Tamime and Robinson, 1999). In recent years, yogurt has also been produced by the incubation of milk with probiotic bacteria, such as *Bifidobacterium*, *Lactobacillus acidophilus*, or *Lactobacillus casei*, designated as probiotic products (Donkor et al., 2007). Yogurt is also known as a health beneficial product from a nutritional point of view because it is rich in calcium, vitamin B, essential fatty acids, and bioactive peptides. The occurrence and level of these bioactive peptides depend on many factors, including starter cultures and production conditions such as fermentation and storage of yogurt. A flow chart showing the production of yogurt is illustrated in Fig. 21.3.

#### 21.3.2 CHANGE IN THE LEVEL OF BCMS DURING FERMENTATION AND STORAGE OF YOGURT

Fermentation is a process step playing an important role in food production. Milk fermented with LAB is widely used for the production of fermented dairy products (i.e., including yogurt) enriched in bioactive peptides. Fermentation causes a drop in pH of milk to required values (i.e., usually 4.5–4.6), leading to changes in the physical–chemical properties of the final products (Beal, 1999). *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* are widely used as starter cultures to produce yogurt and cheese such as Mozzarella and Taleggio. Proteases released from these bacteria can hydrolyze caseins into a number of large casein fragments, which are subsequently degraded by bacterial-derived peptidases into shorter peptides (Nielsen et al., 2009). The bioactive peptides released from caseins during fermentation and storage of dairy products were found to be ACE-inhibitory, antioxidant, and antimicrobial peptides (Donkor et al., 2007; Papadimitriou et al., 2007; Sabeena Farvin et al., 2010, El Hatmi et al., 2016).

In recent years, BCMs, especially BCM5 and BCM7, have been extensively studied in milk and dairy products. Several studies have attempted the identification and quantification of these BCMs in commercial yogurts containing a mixture of *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus*, and other LABs, but neither BCM5 nor BCM7 was reported in these products (De Noni and Cattaneo, 2010). Schieber and Brückner (2000) identified some pro-BCMs in yogurt fermented with *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus* and stored for 21 days, but BCM5 and BCM7 were not detected. These findings suggest that the mixture of starter culture *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus* is not capable of forming BCMs, or such a mixture can produce BCMs, which are subsequently degraded during storage. This hypothesis could be supported by the detection of BCMs in two different products of Taleggio cheese using *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus* as the starter culture (De Noni and Cattaneo, 2010; De Noni et al., 2015). BCM5 and BCM7 have been
FIGURE 21.3
Flowchart of yogurt production (Nguyen, 2016).
Table 21.2 Levels of Beta-Casomorphin (BCM) 5 and Beta-Casomorphin 7 in Milk During Yogurt Processing (Nguyen, 2016).

<table>
<thead>
<tr>
<th>Samples</th>
<th>pH of Fermentation</th>
<th>Storage Time (days)</th>
<th>BCM7 (ng/g)</th>
<th>BCM5 (ng/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unfermented milk</td>
<td>Milk pH (6.5)</td>
<td>0</td>
<td>1.40±0.00</td>
<td>n.d.</td>
</tr>
<tr>
<td>Yogurt</td>
<td>4.8</td>
<td>0</td>
<td>0.29±0.01</td>
<td>n.d.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>0.17±0.01</td>
<td>n.d.</td>
</tr>
<tr>
<td>Yogurt</td>
<td>4.5</td>
<td>7</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

n.d., Not detected.

*Mean value (n = 2) ± standard deviation (SD).

found to degrade during fermentation and storage of yogurt. Spiked UHT milk fermented to yogurt (pH 4.5) was found to be free of BCM5 and BCM7 at any time during storage (Tables 21.2 and 21.3) (Nguyen, 2016). The occurrence of other BCMs in yogurt such as BCM3, BCM4, BCM6, BCM8, BCM9, BCM10, and BCM11 is still unclear; however, some of these BCMs including BCM5 and BCM7 have been found in different types of cheese (De Noni et al., 2015).

Apart from traditional yogurt, today probiotic yogurts are also widely produced. Donkor et al. (2007) used L. casei L26, L. acidophilus L10, or Bifidobacterium lactis B94 added to L. delbrueckii ssp. bulgaricus and S. thermophilus for producing probiotic yogurt. A number of ACE-inhibitory peptides originating from β-CNs were released in these dairy products including β-CN(f193–198), β-CN(f25–29), β-CN(f69–73), β-CN(f84–86), and β-CN(f74–76), but the occurrence of neither BCM7 nor other BCMs was observed. Nonetheless, Jarmolowska (2012) reported the presence of BCM7 in probiotic yogurt using enzyme-linked immunosorbent assay and the level of the measured opioid peptide depended on the type of the product and decreased during storage time (Jarmolowska, 2012).

### 21.3.3 THE ROLE OF INDIVIDUAL YOGURT CULTURES ON DEGRADATION OF BCMS

The choice of bacterial strains plays a crucial role in determining the properties of fermented milk including the peptides’ profile in the final products (Jarmolowska, 2012). S. thermophilus strains can be used as a starter culture in fermented milk. It is an important bacterium in the first stages of fermentation of milk for the production of yogurt. In addition to production of pyruvic acid, formic acid, folic acid, fatty acids, and CO₂ for L. delbrueckii ssp. bulgaricus (Settachaimongkon et al., 2014), strains of S. thermophilus also release a large number of bioactive peptides during incubation (Miclo et al., 2011; El Hatmi et al., 2016). L. delbrueckii ssp. bulgaricus can also be used as a single starter culture for yogurt making. Several studies used L. delbrueckii ssp. bulgaricus SS1 or L. delbrueckii ssp. bulgaricus Y10.13 for fermentation of yogurt and found many peptides derived from β-CNs, such as β-CNf6–14, β-CNf7–14, β-CNf73–82, β-CNf74–82, β-CNf75–82, β-CNf76–180, and β-CNf1–8 (Gobbetti et al., 2000; Papadimitriou et al., 2007). In recent years, the release/degradation of BCM5 and BCM7 during fermentation and storage of yogurt has been reported, and the role of each bacterium has also been demonstrated. In an in vitro study, Hafeez et al. (2013) showed that S. thermophilus CNRZ1066...
## Table 21.3 Recovery of Beta-Casomorphin (BCM) 5 and Beta-Casomorphin 7 as a Percentage of the Theoretical Spiked Value in Milk and Yogurts

<table>
<thead>
<tr>
<th>Sample</th>
<th>Type of Experiment</th>
<th>Bacteria</th>
<th>pH</th>
<th>Storage (days)</th>
<th>BCM5 Amount (ng/g ± SD)</th>
<th>Recovery (%) ± RSD</th>
<th>BCM7 Amount (ng/g ± SD)</th>
<th>Recovery (%) ± RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>UHT milk</td>
<td>Blank</td>
<td>No</td>
<td>Unchanged (6.7)</td>
<td>–</td>
<td>n.d.</td>
<td>–</td>
<td>n.d.</td>
<td>–</td>
</tr>
<tr>
<td>Spiked UHT milk</td>
<td>Quality Control</td>
<td>No</td>
<td>Unchanged (6.7)</td>
<td>–</td>
<td>42.5 ± 0.2</td>
<td>106 ± 1</td>
<td>39.8 ± 0.8</td>
<td>100 ± 2</td>
</tr>
<tr>
<td>Spiked yogurt</td>
<td>Quality Control</td>
<td>–</td>
<td>Unchanged (4.5)</td>
<td>–</td>
<td>39.2 ± 0.5</td>
<td>98 ± 1</td>
<td>40.1 ± 0.4</td>
<td>100 ± 1</td>
</tr>
<tr>
<td>Spiked UHT milk</td>
<td>Acidity medium</td>
<td>No</td>
<td>Acidification to pH 4.5</td>
<td>0</td>
<td>45.4 ± 3.2</td>
<td>113 ± 8</td>
<td>42.7 ± 4.5</td>
<td>107 ± 11</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td>44.5 ± 2.7</td>
<td>111 ± 7</td>
<td>41.8 ± 0.5</td>
<td>105 ± 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>7</td>
<td>42.4 ± 0.5</td>
<td>106 ± 1</td>
<td>41.6 ± 0.2</td>
<td>104 ± 0</td>
</tr>
<tr>
<td>Spiked UHT milk</td>
<td>Yogurt cultures</td>
<td>Yes</td>
<td>Fermentation to pH 4.5</td>
<td>0</td>
<td>n.d.</td>
<td>–</td>
<td>n.d.</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td>n.d.</td>
<td>–</td>
<td>n.d.</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>7</td>
<td>n.d.</td>
<td>–</td>
<td>n.d.</td>
<td>–</td>
</tr>
<tr>
<td>Spiked UHT milk</td>
<td>Yogurt cultures</td>
<td>Yes</td>
<td>Fermentation to pH 4.5</td>
<td>0</td>
<td>n.d.</td>
<td>–</td>
<td>n.d.</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td>n.d.</td>
<td>–</td>
<td>n.d.</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>7</td>
<td>n.d.</td>
<td>–</td>
<td>n.d.</td>
<td>–</td>
</tr>
<tr>
<td>Spiked UHT milk</td>
<td>Yogurt cultures</td>
<td>Yes</td>
<td>Fermentation to pH 4.5</td>
<td>0</td>
<td>n.d.</td>
<td>–</td>
<td>n.d.</td>
<td>n.a.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td>n.d.</td>
<td>–</td>
<td>n.d.</td>
<td>n.a.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>7</td>
<td>n.d.</td>
<td>–</td>
<td>n.d.</td>
<td>n.a.</td>
</tr>
</tbody>
</table>

n.a., Not available; n.d., not detected; RSD, relative standard deviation; SD, standard deviation.

*a* UHT milk spiked with 40 ng/g of BCM5 and BCM7.

*b* Yogurt fermented with Streptococcus thermophilus and Lactobacillus delbrueckii ssp. bulgaricus (pH 4.5) and then spiked with 40 ng/g of BCM5 and BCM7.

*c* S. thermophilus.

*d* L. delbrueckii ssp. bulgaricus.

*e* S. thermophilus and L. delbrueckii ssp. bulgaricus.
completely degraded BCM7 after 4 h incubation; however, when the strain *S. thermophilus* LMD-9 was used, 22 h incubation were needed to achieve complete degradation of BCM7. Similarly, Nguyen (2016) have demonstrated that BCM5 and BCM7 were completely hydrolyzed by either *S. thermophilus* or *L. delbrueckii* ssp. *bulgaricus* after approximately 10 h fermentation (Table 21.3).

The degradation of BCM5 and BCM7 appears only to occur at Pro-X peptide bonds. Two dipeptides suggested being β-CNf60–61 and β-CNf62–63 were identified in commercial yogurts (Kunda et al., 2012). Following the incubation of BCM7 with strains of *S. thermophilus* CNRZ1066 and LMD-9 for nearly a day, Hafeez et al. (2013) also identified several new peptides, including β-CNf60–61, β-CNf62–63, β-CNf64–66, and β-CNf62–66.

However, to date, the extent of BCM hydrolysis by *L. delbrueckii* ssp. *bulgaricus* in in vitro studies and/or in dairy product experiments is still unclear.

### 21.3.4 FACTORS LIKELY TO CAUSE DEGRADATION OF BETA-CASOMORPHINS

Currently, factors causing the degradation of BCMs during yogurt production are unclear. The change in BCM level mainly occurs during fermentation and cold storage. It is well known that peptides containing proline residues are more resistant to hydrolysis by human digestive enzymes, but they can be hydrolyzed by X-prolyl-dipeptidyl aminopeptidase (PepX) derived from bacteria (Donkor et al., 2007; De Noni et al., 2015). BCM5 and BCM7 are proline-rich peptides (Fig. 21.1) and it has been demonstrated that proline residue in the second position on the sequence of BCM7 protects the peptide from hydrolysis at the N-terminal amino acid residue by other bacterial aminopeptidases; however, BCM7 is susceptible to hydrolysis by PepX (Hafeez et al., 2013). Most dairy cultures used for fermentation of milk are LAB that possesses PepX activity (Donkor et al., 2007). The strains *Lactococcus lactis* ssp. *cremoris* and *Lactobacillus helveticus* have been demonstrated to release PepX and completely degrade BCM7 (Kiefer-Partsch et al., 1989; Matar and Goulet, 1996). In vitro studies have shown that *S. thermophilus* produces intracellular and extracellular PepX (Hafeez et al., 2013; Meyer and Jordi, 1987). *L. delbrueckii* ssp. *bulgaricus* has been also reported to produce intracellular and extracellular PepX (Atlan et al., 1990; Miyakawa et al., 1991). Nguyen (2016) found that BCM5 and BCM7 were stable in acidic pH medium (pH 4.5) with no bacteria (Table 21.3), but were susceptible to degradation in the presence of yogurt bacteria. A correlation between PepX activity and the degradation of BCM5 and BCM7 in yogurt fermented by a mixture of *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus* was also found suggesting that PepX activity was a factor promoting the degradation of both peptides during yogurt storage.

### 21.3.5 ROUTINE AND NOVEL ANALYTICAL METHODS FOR THE IDENTIFICATION AND QUANTIFICATION OF BCMS IN DAIRY PRODUCTS

Liquid chromatography coupled to mass spectrometry (LC-MS) is the tool of choice for research concerning the identification and quantitation of ionizable compounds in complex matrices including analysis of small peptides such as BCMS in dairy products (Nguyen et al., 2015b). Because of its versatility, sensitivity, and selectivity LC-MS has found numerous applications in this field both in terms of (1) BCMS analyzed (e.g., from BCM3 up to BCM13) and (2) matrices analyzed (e.g., yogurt as well as milk, human colostrum, infant formula, dried milk, and cheese, among others) (Nguyen et al., 2015b).
Since the late 1990s, reversed-phase high performance liquid chromatography (RP-HPLC) coupled to UV–Vis detection has been used for the analysis of amino acids and small peptides including BCMs in human colostrum, milk, and cheese (e.g., Muehlenkamp and Warthesen, 1996; Jarmołowska et al., 2007; Nguyen et al., 2015b). While this technique is widely available, relatively cheap, and extremely easy to use (i.e., in comparison to MS techniques), it fundamentally lacks analytical specificity. Peptides and organic interferences with similar physical–chemical (and spectrophotometric absorption) properties may coelute with BCMs, resulting either in (1) false positives (i.e., wrong peak assignment) or (2) inaccurate quantitative results (i.e., increased absorption values resulting in an overestimation of the actual BCM content in the samples) (Muehlenkamp and Warthesen, 1996; Cass et al., 2008; Sienkiewicz-Szłapka et al., 2009). Nowadays, detectors such as UV–Vis diode array detectors are available, enabling the UV–Vis spectra to be recorded continuously over the chromatographic run. For increased confidence in the identification of suspect peaks, the UV–Vis spectra of a peak eluting at a certain retention time can be matched against a standard or a reference library. Methods employing UV–Vis adsorption may also lack the sensitivity required to quantify low levels of peptides found in some dairy products. Detection limits of HPLC UV–Vis methods have been reported to span between 0.6 and 2 μg/mL, while, for example, levels of BCMs have been found in the low ng/mL range (Nguyen et al., 2014, 2015a,b).

Technology developments and improvements in the field of liquid chromatographic separation and multistage fragmentation/MS detection have pushed the limits in terms of increased sensitivity, better automation, high throughput, and reduced analysis time resulting in enhanced analytical accuracy along with improved identification criteria to avoid false positives and false negatives. HPLC and the more recent ultraperformance liquid chromatography (UPLC) (for increased chromatographic resolution and speed of analysis) coupled to different ranges of MS technologies have been demonstrated as suitable analytical techniques for identifying and quantifying BCMs in complex dairy matrices at low levels.

A range of configurations from a number of MS vendors are available on the market, where ion trap (IT) and triple quadrupole (QQQ) mass spectrometers are still among the most popular. These systems routinely deliver excellent sensitivity and selectivity at reduced cost. ITs are very useful when operated in data-dependent acquisition mode, where an automated switching between MS and MS/MS during a scan is triggered on the basis of certain threshold criteria set by the operator. For this, the IT is generally set to acquire an MS scan spectra followed by, for example, multiple-stage fragmentation spectra of (1) preselected target ions (e.g., a list of m/z masses corresponding to the parents’ compounds to target) and (2) “top N” intense peaks from the previous MS scan spectra. This allows quantifying and qualifying target analytes, while acquiring information on unknown/nontarget peaks. Examples of BCM analysis using IT in milk and cheese have been reported previously (e.g., Juan-García et al., 2009; Rizzello et al., 2005) and such analytical methods could be easily extended to the analysis of the same peptides in yogurt. QQQ mass spectrometers are particularly useful when operated in multiple reaction monitoring (MRM) mode for unprecedented sensitivity, good selectivity, and low risk of false positives (i.e., through analysis of the MRM ratio between quantifying and qualifying ions). Nowadays, the most advanced QQQ configurations feature a number of combined mass spectra acquisition modes including (1) triggering a product ion scan when a peak is detected by MRM, which enables confirmation of the identities and structures while acquiring quantitative data, and (2) acquiring simultaneously full-scan MS data and quantitative data in MS/MS mode, enabling detection and accurate quantitation of target and “nontarget” analytes (i.e., analytes that are not in a targeted MRM screening method).
along with the full characterization of the background matrix. Examples of BCM analysis using the QQQ system in dairy products including yogurt have been reported previously (e.g., De Noni, 2008; De Noni and Cattaneo, 2010; Nguyen et al., 2014, 2015a; Sabeena Farvin et al., 2010; Toelstede and Hofmann, 2008). Recently, low mass resolution hybrid systems combining linear IT and QQQ MS technology (i.e., known as “Q-traps”) have been introduced on the market. This hybrid configuration features multiple data acquisition modes typical of IT and QQQ systems (e.g., MS scan, product scan, parent scan, neutral loss, MRM, and a combination of them) and novel data acquisition modes including MRM^3 (i.e., also known as MRM-cube). The MRM^3 workflow makes use of data originated from transitions between a parent ion and second-generation product ions. The MRM^3 workflow often provides higher specificity compared to the MRM workflow boosting the selectivity for quantitative assessment of low-level analytes in the presence of challenging coeluting interferences. To the best of our knowledge, the application of the MRM^3 workflow to the analysis of BCMs in dairy matrices has yet to be reported in the literature. However, it is easy to understand how the analysis of BCMs in such challenging matrices could benefit from the MRM^3 workflow, which provides higher specificity and selectivity, drastically reducing the need for extensive sample clean-up by offline solid-phase extraction along with the need to develop complicated chromatographic separations to separate analytes from background interferences.

Accurate mass full-scan MS and MS/MS analyses, either using quadrupole time-of-flight (Q-TOF) and Orbitrap high-resolution MS technologies, are also becoming a routine requirement in many analytical laboratories around the world, and certainly these techniques represent valid alternatives to modern low-resolution IT, QQQ, and Q-trap systems.

Full-scan data acquired through Q-TOF and Orbitrap mass spectrometers enable the accurate mass screening of targeted as well as untargeted analytes. High accuracy and high-resolution MS data are of particular value for confirmation of “known” analytes as well as for the elucidation of “unknown” compounds in complex matrices. This is achieved by combining elemental composition information obtained from high mass accuracy experiments (e.g., number of atoms of carbon, hydrogen, oxygen, and nitrogen in a molecule) with structural information obtained from multiple-stage fragmentation experiments (i.e., limited to HRMS^2 for Q-TOF and HRMS^n for Orbitrap systems). Both Q-TOF and Orbitrap MS systems are appropriate tools for the successful identification and quantification of small peptides in samples containing high amounts of matrix interferences, with modern Orbitrap systems generally providing better resolution and therefore better mass accuracy compared to Q-TOF systems. For example, using HPLC TOF-MS, Toelstede and Hofmann (2008) reported the presence of BCM9 and BCM10 in the water-soluble extract of a matured Gouda cheese. In another study, BCM13 was detected in yogurt using a micro-LC coupled to a TOF mass spectrometer (Kunda et al., 2012). More recently, the peptides BCM5 and BCM7 were analyzed by matrix-assisted laser desorption/ionization time of flight (MALDI-TOF)/TOF MS in hydrolyzates of β-CN variants (A1A1, A1A2, and A2A2) following fractionation by RP-HPLC (Ul Haq et al., 2015).

In recent years, the use of Orbitrap technology for the specific analysis of BCMs in dairy products has also been reported in the literature. For example, Nguyen et al. developed an analytical method to identify and quantify BCM5 and BCM7 in milk extracts (Nguyen et al., 2015a). Analyte detection was achieved using an LTQ Orbitrap XL mass spectrometer operated in single reaction monitoring, high resolution mass spectrometry (HRMS) (60,000 resolution), and HRMS^2 (30,000 resolution) modes for identification and quantitation of BCM5 and BCM7 in dairy products. In the same year, De Noni et al. also published an analytical method using UPLC coupled to Q Exactive Quadrupole-Orbitrap MS for the identification and quantification of BCM3, BCM4, BCM5, BCM6, and BCM7 in a number of cheese samples and their
digestedates (De Noni et al., 2015). Detection of analytes was achieved in a targeted single ion monitoring and data-dependent HRMS2 scan with resolutions of 35,000 and 17,500, respectively.

Regardless of the selectivity, sensitivity, resolution, mass accuracy, or type of mass spectrometer used, ion suppression (e.g., a form of matrix effect) is one of the main problems affecting detection capability, accuracy, and precision of LC-MS (Jessome and Volmer, 2006; Busetti and Swann, 2013). Ion suppression originates in the ion source, where the mobile phase containing the analytes of interest is converted into a spray of charged molecules for subsequent MS analysis. Interfering species (e.g., nontarget peptides and additional ionizable interfering organic molecules) also may coelute with the analytes of interest resulting in a phenomenon known as “matrix effect” (Jessome and Volmer, 2006). This generally leads to two main issues: (1) false negatives (i.e., no peak detection) and (2) inaccurate quantitative results (i.e., loss of accuracy and precision). In the first instance, coeluting matrix interferences may completely suppress the signal of the analyte of interest resulting in a “false negative”; in the second instance, matrix interferences may suppress the signal to a certain degree, leading to an underestimation of the actual concentration of the analytes of interest in the sample. For example, matrix effects were found to be responsible for causing 26% and 40% signal suppression for BCM5 and BCM7 in spiked yogurt, respectively (Nguyen et al., 2014). Given matrix effects are often an intrinsic characteristic of the sample/s analyzed, investigating their occurrence and magnitude during the method development and validation stages is very important; however, this does not guarantee that a similar level of ion suppression/matrix effect will be encountered in the next batch of samples/matrix analyzed. Therefore to consistently overcome matrix effects and achieve accurate and precise quantitative assessment of BCMs, it is paramount to include deuterated homologs in the analytical procedure. For example, using stable isotope-labeled BCMs (i.e., [2H10] BCM5 and [2H10] BCM7) excellent accuracy and precision were achieved in the analysis of quality control samples spiked with BCM5 and BCM7 in milk and yogurt (Nguyen et al., 2014, 2015a). Alternatively, to take into account ion suppression effects, calibration curves should be built into the matrix to be tested (i.e., standard addition method) (De Noni and Cattaneo, 2010; Jessome and Volmer, 2006). For confident detection and quantification of BCMs in complex matrices including dairy products as well as digestates and biological fluids, the use of HPLC and UPLC coupled to low-/high-resolution MS is recommended. To overcome matrix effects and to account for analyte recoveries during sample extraction and purification, the use of deuterated surrogates or matrix-matched calibration curves is also strongly recommended.

This chapter has provided a general overview on the origin and biological activity of BCMs. According to the scientific literature, the key processing conditions affecting the degradation of BCMs are fermentation and cold storage. The degradation of BCMs, especially BCM5 and BCM7, is likely a result of yogurt bacteria derived PepX activity. To understand the role of yogurt bacteria that cause the degradation of BCMs, future research directions should use a number of various strains of yogurt starter cultures to assess the capability of BCM degradation. In addition, future research should also use mutant strains of S. thermophilus and L. delbrueckii ssp. bulgaricus with deficiency in PepX to elucidate the main factors affecting the degradation of BCMs.

The development and application of analytical methods employing liquid chromatography coupled to hybrid tandem MS and/or high-resolution MS along with the methodical use of deuterated homologs
is strongly recommended to accurately identify and quantify BCMs in complex dairy products. The ability to confidently analyze small proteins and peptides with known/suspect health impacts in food will enable the development of a new paradigm in directly measuring and validating molecules and, at the same time, use these foods for clinical studies.

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REFERENCES


22.1 INTRODUCTION

Lactose (β-d-galactopyranosyl-(1→4)-d-glucose) is the most abundant constituent after water in bovine milk. Lactose is also the main carbohydrate in the milk of virtually all mammalian species. Mono- and oligosaccharides are also present in the milk of most species in small quantities. Technologically, lactose is the most important carbohydrate in milk and is in fact important for many dairy products, for example, it is crucial in the lactic acid fermentation of cheese, yogurt, and other acid-coagulated dairy products (Walstra et al., 2005). Furthermore, lactose is applied in a wide range of dairy and nondairy food products, as well as in nonfood products (Holsinger, 1997). Lactose is also converted into lactose derivatives, for example, galactooligosaccharides (GOS), lactulose, lactitol, and lactobionic acid (Gaenzle et al., 2008). Additionally, because lactose is a reducing carbohydrate, it can participate in the Maillard reaction, which can occur in heated dairy products, particularly when heated at sterilization conditions (Walstra et al., 2005).

When considering the different constituents of yogurt and fermented milk products, lactose is primarily considered as a source of carbohydrate for lactic acid bacteria. However, lactose has various other effects as well, as will be described in this chapter. Lactose affects yogurt texture and is a source for the formation of oligosaccharides during fermentation. Furthermore, specific cultures can be used to ferment lactose to increase the sweetness of yogurt without the necessity for added sweeteners. In addition, lactose digestion in yogurt by people suffering from lactose intolerance is a topic of interest, because in this product format lactose is actually tolerated by lactose mal-absorbers or lactose-intolerant people. In this chapter, the various roles of lactose are highlighted and discussed.

22.2 PROPERTIES OF LACTOSE

One important property of lactose is its mutarotation behavior, that is, two anomers of lactose are observed, α-lactose and β-lactose; these have a specific optical rotation $[\alpha]_D^{20}$ of +89.4 degrees and +35 degrees, respectively (Walstra and Jenness, 1984; Holsinger, 1997). Conversion between these anomers occurs via the open chain form of the glucose moiety of lactose. An equilibrium will establish at ~37% α-lactose and 63% β-lactose, that is, at $[\alpha]_D^{20} = +55.3$ degrees (Walstra and Jenness, 1984; Holsinger, 1997). Increases in lactose concentration and temperature shift the equilibrium optical rotation to lower values (i.e., to a higher percentage of β-lactose), whereas pH does not affect the
equilibrium value, but does affect the rate at which the equilibrium established; the minimum in mutarotation rate occurs at pH 5, whereas at pH < 2 and pH > 7 very rapid mutarotation occurs (Walstra and Jenness, 1984; Holsinger, 1997). Mutarotation rate also increases with temperature, that is, almost threefold for every 10°C (Walstra and Jenness, 1984). Lactose mutarotation rate is strongly enhanced by the presence of milk salts and is approximately twofold higher in a simulated milk ultrafiltrate compared to that in water (Haase and Nickerson, 1966). Lactose solubility is strongly affected by the different anomers and is rather low compared with many other mono- and disaccharides. At room temperature, the solubility of lactose is ~20 g of anhydrous lactose per 100 g of water, which increases to ~30, 60, or 100 g of lactose per 100 g of water at 40, 60, or 80°C, respectively. The solubility of lactose can exceed considerably before crystallization occurs. This supersolubility of lactose before crystallisation occurs is typically equal to solubility at a temperature 30°C higher (Paterson, 2009). For understanding lactose solubility in milk rather than in water, the effect of other constituents such as proteins and salts should be considered. Proteins can have high water-binding capacity (Walstra et al., 2005) and can thus reduce lactose solubility. Reduced solubility of lactose in the presence of lactic acid or lactate (Smart, 1988; Smart and Smith, 1992) can also be caused by a fraction of the water being unavailable as a solvent for lactose. Minerals can further affect lactose solubility through affecting the hydrogen bonding structure of water and hence enhancing or reducing solvent quality (Huppertz and Gazi, 2016).

### 22.3 SOURCES OF LACTOSE IN YOGURT

In most cases, the major source of lactose in yogurt and related products is milk or milk powder. Milk typically contains 4.0%–5.0% lactose. In many cases, dairy ingredients are added to milk to increase protein content prior to yogurt manufacture. Particularly, skim milk powder (SMP), whey protein concentrate (WPC), and milk protein concentrate (MPC) are popular ingredients. SMP contains ~30%–35% protein and 50%–55% lactose on a dry matter basis, and protein fortification thus also involves a considerable increase in lactose content. For WPC and MPC, the amount of added lactose is dependent on the type added. Low-protein ingredients, such as WPC35, will result in considerable increases in lactose content, whereas the use of high-protein ingredients, such as WPC80 or MPC80, allows protein fortification without large increases in lactose content. Caseinates, which are also used for protein fortification, contain only very low levels of lactose and thus do not contribute largely to the lactose content of yogurt. In addition to the addition of ingredients, membrane filtration techniques such as ultrafiltration or microfiltration may also be used to increase protein content. In this case, however, protein content is increased and the lactose content remains constant.

### 22.4 LACTOSE FERMENTATION IN YOGURT

As outlined earlier, one of the key roles of lactose is its conversion to lactic acid by lactic acid bacteria during yogurt production. Yogurt is traditionally prepared with a mixture of the thermophilic homofermentative Streptococcus thermophilus and Lactobacillus delbrueckii ssp. bulgaricus, both of which can metabolize lactose and glucose hydrolyzed from lactose, but they do not metabolize galactose. In several countries, the name “yogurt” is only allowed for those products that are produced with cultures containing both strains (Sieuwerts et al., 2008). Although the two microorganisms are able to grow
individually in milk, they have a symbiotic interaction called “protocooperation” in mixed cultures, which means they are mutually beneficial during fermentation (Sieuwerts et al., 2008; Tamime, 2002). Lactose is the primary carbon and energy source for lactic acid bacteria in yogurt production. The lactic acid bacteria convert lactose into lactic acid, which gives yogurt the characteristic acidic taste. In the process of yogurt production, around 20%–40% of lactose present in milk is transformed into lactic acid, and the content of lactic acid in yogurt is around 0.9% (Shiby and Mishra, 2013; Tamime and Robinson, 1999). Lactose fermentation by lactic acid bacteria may be homofermentative or heterofermentative, depending on the species, the substrate, and the environmental conditions (Mayo et al., 2015). Homofermentative pathways generate lactic acid as the main end-product, whereas in heterofermentative metabolism, lactic acid is further metabolized, which results in ethanol, carbon dioxide, or acetic acid (Endo and Dicks, 2014). In yogurt manufacture, homofermentative fermentation is typically observed. During fermentation, the glucose moiety from lactose is fermented to lactic acid, whereas the galactose moiety is not further metabolized.

A publication by Sørensen et al. (2016) highlighted that remodeling the carbohydrate metabolism in L. delbrueckii ssp. bulgaricus and S. thermophilus can be used to actually enhance sweetness. Normally, as outlined previously, glucose is metabolized, whereas the less sweet galactose is not further metabolized. However, by selection of spontaneously occurring mutants, a consortium could be created with drastically altered carbohydrate metabolism. In this case, mutants that utilize galactose rather than glucose and secrete glucose rather than galactose were selected. As a result, the combined strains could ferment milk to form yogurt with enhanced sweetness because of high glucose levels. In addition, virtually no lactose remained in products, whereas normally 60%–80% of lactose remains intact (unmetabolized) (Sørensen et al., 2016). Such strains thus offer the possibility of producing yogurts with enhanced sweetness without the requirement for added sweetness.

22.5 OLIGOSACCHARIDES IN YOGURT

As outlined in the introduction section, milk also contains some oligosaccharides. Whether these are fermented during yogurt manufacture or whether they are retained in yogurt is thus far unknown. In addition, however, analysis of various yogurt and fermented milk products indicates that oligosaccharides are present at higher levels in these products than in milk. Toba et al. (1982, 1983) detected allo-lactose and galactobiose in commercial yogurt samples, at concentrations up to 0.1%. Their conclusions that these oligosaccharides were formed by the lactases in lactic acid bacteria during yogurt manufacture were confirmed in experiments where oligosaccharide was found to increase during yogurt manufacture and storage (Toba et al., 1983). A wide range of oligosaccharides in commercial yogurts was also found by Saito et al. (1998) and Martinez-Villaluenga et al. (2008). The latter authors also observed differences in oligosaccharide concentration and type based on the cultures used. Yogurt containing Bifidobacterium and drinking yogurt containing Lactobacillus casei were found to contain higher levels of oligosaccharides than yogurt prepared using traditional cultures (Martinez-Villaluenga et al., 2008). Lamoureux et al. (2002) prepared oligosaccharide-enriched yogurt using traditional yogurt cultures in combination with bifidobacteria, wherein oligosaccharides were first produced during a preincubation step for 90 min at 50°C with the bifidobacteria, followed by yogurt production during which oligosaccharide content increased further, up to 0.6%. Oligosaccharide content subsequently remained constant during storage for up to 28 days (Lamoureux et al., 2002).
Considerably higher levels of oligosaccharides are found in yogurt and fermented milk products when lactose is hydrolyzed before or during yogurt manufacture with lactases. Toba et al. (1986) reported that levels of oligosaccharides up to 20 times higher than in control yogurt, reaching a maximum of 1.2%, could be achieved by adding a lactase with the starter culture. Vénica et al. (2015) also showed that the inclusion of a lactase during yogurt manufacture resulted in oligosaccharide levels up to 0.7% in stirred yogurt prepared from a yogurt mix originally containing 7% lactose. GOS levels were found to be stable during storage of the product for up to 21 days, indicating that they were not metabolized and that using such approaches for preparing oligosaccharide-enhanced yogurt could be a viable option.

22.6 EFFECT OF LACTOSE ON YOGURT STRUCTURE

The main effect of lactose on yogurt structure is through the aforementioned formation of lactic acid as a fermentation product from lactose and the concomitant acidification. As a result of the reduction in pH, the colloidal stability of the milk proteins is reduced and, once steric and electrostatic repulsion is overcome, aggregation of protein particles can occur. In yogurt manufacture, milk is typically preheated intensively and initial aggregation involves aggregates of denatured whey proteins (which can also include some κ-casein) and casein micelles containing denatured whey proteins on the surface. In addition, protein-covered fat globules will also participate in these aggregation reactions. Such aggregation typically commences around pH 5.2–5.6, depending on composition and preheating conditions and intensity. Following initial aggregation, growth of aggregates will continue until eventually a self-supporting network is formed, which creates the desired texture, structure, and stability of yogurt.

In addition to the primary role of lactose in yogurt structure and texture as an essential source required for lactose acid product, lactose also has a secondary role in yogurt structure and texture. Niki and Motoshima (2006) carried out a series of studies in which acidic milk gels were prepared using the acidulant glucono-δ-lactose of casein micelle suspensions containing various levels of lactose (0–0.125 mol/L) and observed two clear trends: (1) the pH at which increases in storage modulus (\(G'\)) occurred during acidification of casein micelles suspensions, as a result of acid-induced aggregation of casein micelles, increased with increasing concentration of added lactose, and (2) the maximum \(G'\) value of the acid gels of casein micelle suspensions increased approximately threefold and the gel firming rate more than twofold with increasing concentration of added lactose from 0 to 0.125 mol/L. Further experiments by Niki and Motoshima (2006) also highlighted that these effects of lactose concentration on acid gelation of casein micelles were particularly notable when acidification was carried out at temperatures >20°C, whereas at lower temperatures, effects were less notable. The observed effects were explained by Niki and Motoshima (2006) based on the effect of lactose on solvent quality. The presence of lactose was expected to enhance the hydrogen-bonding structure of water, hence making exposure of hydrophobic segments of proteins less favorable and enhancing hydrophobic interactions between proteins.

The effects of lactose on acid gel structure were examined further by Meletharayil et al. (2015, 2016). In a study on the acid gelation properties of milk protein concentrate solutions (8% protein), Meletharayil et al. (2015) noted considerable differences in acid gelation characteristics of MPC solutions containing different protein:lactose ratios, as well as differences in gel firmness and gel structure. Gels prepared from suspensions of MPC80 were found to be considerably less firm and more porous compared to those from SMP. Reduced lactose content was suggested as one of the possible
Contributors to the findings. The role of lactose was evaluated in more detail by Meletharayil et al. (2016) in a study on acid gelation of SMP and MPC suspensions, which were standardized to 4% protein and different lactose contents. In these studies, it was observed that, with increasing lactose content, heat-induced dissociation of caseins and Ca-ion activity of samples increased. Furthermore, the pH at which acid-induced gelation commenced increased, as did storage modulus and water-holding capacity of the gels, whereas porosity of the gels decreased with increasing lactose content (Meletharayil et al., 2016). These recent findings thus confirm that the findings of Niki and Motoshima (2006) in casein micelle suspensions are also relevant in the case of yogurt manufacture, where both caseins and whey proteins are present and the latter are denatured.

Furthermore, lactose hydrolysis can also have notable effects on yogurt structure. When yogurt was prepared from the yogurt mix preincubated with lactase, it was found that acid production was enhanced, and as a result acid-induced coagulation occurred more rapidly, gel strength was decreased, and whey expulsion was increased (Nagaraj et al., 2009). Schmidt et al. (2016) also observed reduced viscosity when lactose was hydrolyzed prior to or simultaneously with fermentation, but found such effects to be dependent on both yogurt type (regular or Greek style) and culture used. Culture effects appeared to be related to the effect of lactose hydrolysis of acidification rate, that is, in cases where lactose hydrolysis was found to increase acidification rate, reductions in viscosity were observed, whereas in cases where acidification rate remained unaffected, lactose hydrolysis was not affected strongly (Schmidt et al., 2016). Effects may be explained based on the findings of Vasbinder et al. (2003) that incubation time after coagulation has commenced is crucial in achieving gel firmness, possibly because of formation of disulfide bonds between particles. Hence, faster acidification as a result of lactose hydrolysis (Nagaraj et al., 2009; Schmidt et al., 2016) can reduce the time available for such crosslinking to form. Interestingly, Schmidt et al. (2016) also found that lactose hydrolysis tended to increase exopolysaccharide production, a finding that is most certainly worth exploring further.

### 22.7 LACTOSE INTOLERANCE AND YOGURT

Lactose intolerance, or lactose malabsorption, refers to the inability of adults or children to digest lactose. Lactose-intolerant people lack sufficient intestinal lactase activity to digest the lactose present when consuming (e.g., milk or other dairy products). As a result, the undigested lactose enters the colon, where it is fermented by resident microflora, resulting in gastrointestinal symptoms, including flatulence, bloating, and diarrhea. Particularly in non-Caucasian children and adults, the prevalence of lactase deficiency can be higher than 50%, because of a genetically programmed loss of lactase production after weaning.

Whereas consumption of milk products is not possible for lactose-intolerant populations because of the aforementioned gastrointestinal symptoms, such populations often have long histories of consuming yogurt without experiencing these symptoms (Renner, 1997; Solomons, 2002; Savaiano, 2014). This at first would seem counterintuitive, since only part of the lactose is fermented in yogurt manufacture and most lactose actually remains intact. Since yogurt in many cases is fortified with ingredients such as SMP or WPC, it may even have higher residual lactose concentration than milk. Hence, reduced gastrointestinal symptoms for lactose-intolerant people after consumption of yogurt compared to milk thus cannot be related to the lower lactose content of yogurt. A direct comparison study of yogurt and milk with the same concentration of lactose clearly highlighted that lactose in yogurt is better digested than that in milk (Kolars et al., 1984).
A series of animal and human studies performed since the 1980s has highlighted that it is in fact the presence of live cultures in yogurt that aids the digestion of lactose in the intestine (Montes et al., 1995; Lin et al., 1998). Similar effects were observed for fresh yogurt from full-fat, low-fat, and lactose-hydrolyzed yogurt (Marteau et al., 1990; Rosado et al., 1992). In contrast, for heat-treated yogurt, in which cultures are dead and enzymes inactivated, typical gastrointestinal symptoms associated with lactose ingestion by lactose-intolerant people are observed (Rizkalla et al., 2000; Pelletier et al., 2001). To achieve sufficient lactase activity in the intestine, it is important that the lactases survive the conditions of the gastrointestinal tract, particularly its acidic environment, which for adults will be in the range pH 1.0–2.5. At postfermentation pH ∼4.0–4.5 in yogurt, lactase activity is very low and if pH reaches <3.0, lactase activity may be irreversibly lost. However, the high buffering capacity of yogurt helps maintain stomach pH comparably high (Martini et al., 1987) and allows passage of some lactase activity into the intestine, even though some bacteria may lose viability (Pochart et al., 1989).

When the bacterial cells enter the intestine, pH is increased to ∼6.5–7.5 in the duodenum, which is close to optimal for residual lactate. Hence, with lactase activity restored, lactose can be hydrolyzed before it can reach the colon. It has been reported that the presence of bile can increase lactase activity in vitro (Gilliland and Kim, 1984), which may be caused by increased permeability of the cells, thus allowing more lactose to enter the bacterial cells. Lactose digestion in vivo appears to be rather universal for yogurts prepared with typical yogurt cultures (Martini et al., 1991b). Lactase activity was shown to be strongly correlated to the concentration of bacterial cells and a 10-fold reduction in cell count makes residual lactase activity insufficient (Lin et al., 1991). Given this importance of the presence of sufficient live cultures, it may be expected that fresh yogurt is more efficient than aged yogurt, but such studies have not been reported to date. Consumption of a meal with yogurt was not found to affect lactase activity and lactose digestion from yogurt (Martini et al., 1991a).

22.8 CONCLUSIONS

From the foregoing it is clear that the role of lactose in yogurt is beyond that of a carbohydrate source readily available for fermentation by lactic acid bacteria into lactic acid. Lactose can also be a precursor for glucose, thereby enhancing sweetness, or prebiotic oligosaccharides. Furthermore, the presence of residual lactose in yogurt enhances structure and texture through enhancing protein interactions. Surprisingly, lactose in yogurt is far better digested by lactose-intolerant people because of the presence of live cultures aiding lactose digestion in the intestine.

REFERENCES


BACTERIA IN YOGURT AND STRAIN-DEPENDENT EFFECTS ON GUT HEALTH

23

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23.1 INTRODUCTION

To define the focus of this chapter, it appears relevant to introduce some notes about two important words in the title: bacteria and yogurt.

According to Codex Alimentarius (CODEX STAN 243-2003), yogurt is defined as a category of fermented milk product characterized by two specific starter cultures used for its fermentation: *Streptococcus thermophilus* and *Lactobacillus delbrueckii ssp. bulgaricus*.

In this chapter fermented milk is defined as a milk product obtained by fermentation of milk, which contains starter microorganisms that shall be viable, active, and abundant in the product through to the expiry date. This requirement also applies to yogurt starter bacteria; the same standard contains the following definitions:

- **Alternate Culture Yogurt**: Cultures of *Streptococcus thermophilus* and any *Lactobacillus* species.
- **Acidophilus Milk**: *Lactobacillus acidophilus*.
- **Kefir**: Starter culture prepared from kefir grains, *Lactobacillus kefiri*, species of the genera *Leuconostoc*, *Lactococcus*, and *Acetobacter* growing in a strong specific relationship. Kefir grains constitute both lactose-fermenting yeasts (*Kluyveromyces marxianus*) and nonlactose-fermenting yeasts (*Saccharomyces unisporus, Saccharomyces cerevisiae, and Saccharomyces exigua*).
- **Kumys**: *Lactobacillus delbrueckii ssp. bulgaricus* and *Kluyveromyces marxianus*.

Microorganisms other than those constituting the specific starter culture(s) specified previously may be added.

As regards the statement that “microorganisms shall be viable, active, and abundant,” the Codex standard indicates $10^7$ as the minimum total amount of the sum of microorganisms constituting the starter culture (cfu/g in total) and a minimum of $10^6$ cfu for a microorganism listed on the label.

In this chapter, data on the beneficial action on health because of yogurt starter cultures *S. thermophilus* and *L. delbrueckii ssp. bulgaricus* are reviewed avoiding any reference to alternate yogurt in the Codex or to so-called probiotic yogurt, i.e., fermented milk in which *Lactobacillus* and/or *Bifidobacterium* usually isolated from the intestinal environment are added to exert an effect beneficial to consumers’ health.

Furthermore, the nutritional health effects of yogurt as a source of proteins will be not addressed here.
23.2 YOGURT BACTERIA BETWEEN TAXONOMY AND HISTORY

Bacteria used to ferment milk to obtain yogurt belong to thermophilic, bile-sensitive species of lactic acid bacteria, not ideally suited for survival in the human gut.

*S. thermophilus* was described in 1919 by Orla Jensen in his famous book *The Lactic Acid Bacteria*, a milestone in the history of dairy microbiology. He noticed the thermophilic traits of this species: “*Streptococcus thermophilus* is the most frequently occurring streptococcus in pasteurized milk,” “is in a state of good viability as it is certainly not killed below 80°C,” and “grows as a rule at 50°C.”

Therefore this species has been described as heat loving and heat resistant but definitely not belonging to the intestinal environment.

The current taxonomic position of *S. thermophilus* has been described by Schleifer et al. (1991) and is now well consolidated and taxonomically stable.

More troubling and complex is the history of the nomenclature of the rod bacterium nowadays identified as *L. delbrueckii* ssp. *bulgaricus* but historically named *Lactobacillus bulgaricus* (and this short name will be used throughout this chapter).

Originally described (but not in so clear a way as for *S. thermophilus*) by Orla-Jensen (1919) and Rogosa and Hansen (1971), it missed the status of species after Weiss et al. (1983); genetic data prompted these authors to reclassify the yogurt rod bacterium as *L. delbrueckii* ssp. *bulgaricus*, which is its current taxonomic position.

Allotted to the “genus” *Thermobacterium* by Orla-Jensen, these strains were often misclassified; in Table XXVII of the Orla-Jensen book the two *Thermobacterium* isolated from English and Bulgarian yogurt were reported to produce “inactive” (meaning DL) isomers of lactic acid; they then were possibly what we now call “*Lactobacillus helveticus*.”

This misclassification between *L. bulgaricus* and *L. helveticus* is also present in the always cited work of E. Metchnikoff, the first scientist who established a role for yogurt bacteria as “health promoters.”

At the beginning of the 20th century, Metchnikoff, who received the Noble Prize for his study on immunology, was turning his area of research from “immunity” to the more widespread “resistance” concept.

In his widely cited (but probably not so well-known) *The Prolongation of Life* (Metchnikoff, 1907), devoted to this subject, Metchnikoff provided a detailed view of his ideas on aging and senility. This book is more a philosophical “essay” than a paper on intestinal or food microbiology and among the 34 chapters only three deal with bacteria and the intestine.

In these three chapters, he suggested that the large intestine could exert a negative role for the health of humans: “...it attended with disadvantages that may shorten the actual span of life. The accumulation of waste matter, retained in the large intestine for considerable periods, becomes a source for microbes and produces fermentation and putrefaction and is harmful for the organism.”

He soon realized that it is not possible to have an “aseptic” colon to prevent putrefaction, and he suggested to “disinfect the content of the large intestine.”

After several unsuccessful experiments made in vivo using pure lactic acid (he did not have to submit his clinical protocol to an ethical committee), he turned to the Swiss Prof. Massol, who sent to Metchnikoff a sample of “Bulgarian yahourt.” The team of Metchnikoff (Drs. Cohendy and Michelson) isolated from this product a strain they named “*Bulgarian bacillus*,” as a reminder of its geographical origin, to be used to ferment milk to be administered to subjects exhibiting putrefactive-type fermentations in their colon. This administration caused a marked decrease in the fecal amount of products of putrefaction. In addition, Metchnikoff found that a predominantly Gram-positive flora was established and that the *Bulgarian*
bacillus persisted in stools within 8–12 days after the start of treatment (we do not know how exactly he identified this strain among the lactobacilli present in the fecal samples). Quite luckily the strain is still available in ATCC under the number 521, but its taxonomic position is one of L. helveticus (previously Lactobacillus jugurti) and not of L. bulgaricus! This is in agreement with the original description of this strain provided by Metchnikoff himself, which was reported to be a bacterium able to produce 25 g/L of lactic acid when grown in milk. This high level of acidity is not typical of “bulgaricus” strains as we define them today and more closely resembles the values recorded for L. helveticus strains (Kandler and Weiss, 1986).

23.3 INTESTINAL VIABILITY OF YOGURT BACTERIA

The taxonomic and environmental notes of the previous section have clearly assigned the status of nonintestinal organisms to both yogurt bacteria, with little chance of surviving during gut transit. However, assessing the viability of these bacteria through the digestive tract may be relevant to evaluate their potential to deliver some beneficial effects for the wellbeing of the consumer.

Intestinal survival of bacteria contained in fermented milk has been challenged since the beginning: among the most intriguing results reported by Metchnikoff (1907) was the evidence he obtained that: “lactic acid bacteria present in products such as yogurt, …” were not able to survive into the human gut as well as, on the contrary, his Bulgarian bacillus was.

Since these pioneering data, a number of studies reporting the fate of L. bulgaricus and S. thermophilus in the human gut clearly showed that their survival is very low in the upper part of the gastrointestinal tract. After this negative starting point, the ingested bacteria have to survive during transit through the large intestinal tract.

Survival has been generally measured by plate counts of stool samples; results of the assessment of viability of L. bulgaricus and S. thermophilus ingested by humans and with a full range of information on the amount of each species are summarized in Table 23.1, while further comments on the in vivo assessment of viability of yogurt starter cultures are reported here.

| Table 23.1 Survival of Yogurt Starter Cultures as Measured in Human Subjects After Yogurt Ingestion and Measured by Plating Counts |
|---|---|---|---|---|---|---|
| No of Treated Subjects | Yogurt Intake per Day | Daily Amount of Streptococcus Cells/g Feces | Survival of Lactobacillus Cells/g Feces | Daily Amount of Lactobacillus Cells/g Feces |
| Brigidi et al. (2003) | 5 | 250 g | $10^8$–$10^{11}$ | $4 \times 10^5$ | NR |
| del Campo et al. (2005) | 114 | 375 g | $10^{10}$ | None | $10^9$ |
| Mater et al. (2005) | 13 | 125 g | $8 \times 10^{10}$ | $6.3 \times 10^4$ | None |
| Eli et al. (2006) | 20 | 250 g | $5 \times 10^9$ | None | $6 \times 10^9$ |
| | | | | | $7.2 \times 10^4$ cfu/g |
| | | | | | Log 3–5.5 |

NR, Not reported.
Only papers containing data on the counts of bacteria administered in yogurt are summarized in this table.
In 1987 Conway et al. reported the survival in vivo and in vitro of four strains of lactic acid bacteria administered as pure cultures. Among these strains there was one strain each of *L. bulgaricus* and *S. thermophilus*, while the other two were classified as intestinal isolates of *L. acidophilus*, although one of them, the strain named ADH, was later reclassified as *Lactobacillus gasseri* (Raya and Klaenhammer, 1992).

The in vitro results (survival of bacteria in phosphate-buffered saline at various pH, as determined by viable counts) were negative for *S. thermophilus* (no survival at pH 3), while they were somewhat better for *L. bulgaricus*, even if the best performers were the two intestinal isolates of *L. acidophilus*.

For the in vivo test, a nasogastric tube was used and the *Lactobacillus* suspension (1.0 mL) was introduced into the stomach of volunteers via the tube. Then, 0, 20, 40, 60, 90, 120, and 140 min after the administration of the culture, a 25-mL aspirate was collected and plate counted.

The same procedure in the same subjects was then repeated, but adding bacterial suspension in skim milk; this significantly raised pH and enhanced survival.

As an overall conclusion of these pioneering data, it appears that *S. thermophilus* has very little chance of surviving during stomach transit, while *L. bulgaricus* is a little better but definitely not at the same level as lactobacilli known to be normal inhabitants of the gut.

It appears worthwhile to provide details on a number of studies (see also Table 23.1) performed with human subjects consuming yogurt but not pure starter cultures (Pedrosa et al., 1995; Brigidi et al., 2003; del Campo et al., 2005; Mater et al., 2005; Elli et al., 2006; García-Albiach et al., 2008; Ballesta et al., 2008).

As a general rule, in the large majority of these trials *L. bulgaricus* cells were detected, albeit at low levels, in stools of all the studies in which both species have been investigated, while viable cocci were recovered only in two studies (Brigidi et al., 2003; Mater et al., 2005). However, in the latter case, the number of viable cells present in yogurt was $8 \times 10^{10}$ per each strain, an amount that is not generally present in commercial yogurt, while in the first one there are doubts about the methodological soundness of the identification procedure used (see later).

However, the results of Mater et al. (2005) could be explained not only by the higher amount of ingested cells but also by differences in the used strains, a strain-dependent survival.

It appears worthwhile to also comment on the work of Ballesta et al. (2008), published in Spanish, reporting a crossover trial in which 30 adult volunteers were fed fresh yogurt for 15 days, three times a day (for a total of 375 g of yogurt). The same group of subjects, after a washout period of 15 days, consumed the same amount of pasteurized yogurt for 15 days.

Selective plate counts showed the presence of *L. bulgaricus* only in 0.7% of more than 200 stool samples analyzed, while *S. thermophilus* was never found.

However, the methodologies used to detect cells of yogurt starter cultures in stools have a paramount relevance and some of the results reported in this kind of study are worthwhile discussing in detail.

In the human trial of Brigidi et al. (2003), five healthy subjects ingested 250 g of yogurt daily for 10 days and stools collected from all healthy subjects were analyzed for the quantitative detection of *S. thermophilus* but not *L. bulgaricus* by a culture-independent polymerase chain reaction (PCR) method.

ThI and ThII were used as described by Tilsala-Timisjarvi and Alatossava (1997), to discriminate *S. thermophilus* from mesophilic lactococci, allowed to enumerate during yogurt intake an increase of streptococcal population up to a maximum of $4 \times 10^{5}$ cells/g feces after 3 days since the beginning of the trial. This concentration, the highest reported among the published results of human trials, remained constant during the remaining feeding period of 7 days and slowly decreased below the detection limit of the direct PCR analysis 9 days after treatment suspension.
Specificity of the primers used to detect *S. thermophilus* was tested against 13 bacterial strains belonging to other intestinal species such as *Streptococcus* (nowadays *Enterococcus*) *faecium*, *L. acidophilus*, *Escherichia coli*, *Enterococcus faecalis*, *Clostridium perfringens*, *Bacteroides fragilis*, *Bifidobacterium bifidum*, *Bifidobacterium breve*, *Bifidobacterium infantis*, and *Bifidobacterium longum*.

Other authors (Elli et al., 2006), however, noticed that the use of primers ThI and ThII could not discriminate between *S. thermophilus* and *Streptococcus salivarius*, as previously observed also by the authors who described the primers (Tilsala-Timisjarvi and Alatossava, 1997).

The use of primers ThI and ThII could then lead to incorrect results if not supported by a further strain-specific recognition, and also could result in an overestimation of the presence of thermophilic streptococci in fecal samples.

It is concluded that the assessment of yogurt culture survival in the human gut, even if it is of paramount relevance for the evaluation of health benefits, is not an easy task, since it requires well-validated molecular tools and has to be investigated in depth.

An additional observation comes from an accurate literature search that points out indications for a strain specificity of intestinal survival rate of yogurt cultures as well as potential beneficial actions possibly exerted by different strains of yogurt cultures. In 2007, our laboratories detected, by means of pulse field gel electrophoresis analysis (Tosi et al., 2007), differences in the chromosomal arrangements of several strains taxonomically identified, by using 16S RNA sequences, as *S. thermophilus*, supporting a potential difference also in phenotypic behavior.

The presence of different subgroups within the species *S. thermophilus* was further confirmed by Yu et al. (2015) by sequencing 10 housekeeping genes of 239 of *S. thermophilus* plus 18 reference strains.

Phylogenetic analysis revealed the presence of five lineages within the *S. thermophilus* isolates; these groups appear to have some association, even if not really significant, with geographic origin but, quite surprisingly, revealed no relationships with the types of fermented dairy product from which they were isolated.

Strain-specific survival during gut transit has indeed also been shown by recent reports for *L. bulgaricus*, but these studies were carried out using pure cultures and not fermented milk.

Vázquez et al. (2013) screened a collection of 100 isolates of *L. bulgaricus* isolated from homemade yogurt in rural Bulgarian areas. A preliminary in vitro assessment for deoxycholic acid and pH resistance allowed the authors to identify one strain able to tolerate 3% deoxycholate and pH 3.

This strain was then assessed for its survival and effects on short chain fatty acid production in 20 healthy volunteers (500 mL/day during 15 days). While denaturing gradient gel electrophoresis analysis of DNA extracted from stools of treated subjects at the base demonstrated no significant changes in the qualitative composition of gut microbiota, the presence of a DNA band corresponding to *L. bulgaricus* was observed in all 20 volunteers, but the strain was recovered in a viable form in one subject only. These authors focused their attention on *L. bulgaricus* only and did not check the survival of *S. thermophilus*.

The fate of *S. thermophilus* and *L. bulgaricus* has been reported by García-Hernández et al. (2012a,b) in two in vitro and in vivo reports. The in vitro trial of García-Hernández et al. (2012a) consisted of inoculation of fecal samples with a yogurt starter culture, while that of the in vivo García-Hernández et al. (2012b) experiment was realized by feeding 30 healthy volunteers aged between 25 and 65 in a study that lasted for 11 weeks. The daily dose of yogurt in the intervention period (4 weeks) was 250 g of natural yogurt.
In both experiments, these authors used a direct viable count (DVC) method, combined with fluorescent in situ hybridization (FISH) for the specific detection of viable cells of yogurt starter cultures. In the in vitro trial, cultures of yogurt starter bacteria were incubated for 7 h in the presence of the DNA-gyrase inhibitor; the number of viable cells was obtained by the enumeration of specific hybridized cells that were elongated at least twice their original length for *Lactobacillus* and twice their original size for *Streptococcus*.

In the in vivo trial, a total of 186 samples were analyzed with a detection limit for both viable bacteria for the DVC/FISH technique of $10^3$ cells/g of feces. The authors reported no viable cells of *L. delbrueckii* ssp. *bulgaricus* by this technique in any of the fecal samples analyzed, while there was detection and count of viable *S. thermophilus* in fecal samples.

These results, obtained with a peculiar technique, conflict with the remaining literature and are still to be confirmed.

The presence of a strain-specific ability of *S. thermophilus* to survive to intestinal transit was also assessed in vitro by Uriot et al. (2013) by means of TIM, an in vitro dynamic gastric and small intestinal model; 30 *S. thermophilus* strains were preliminary assessed for the presence of urease, small heat shock proteins, and decarboxylase functions of amino acids. The rationale of this investigation was that all of them were functions that could be putatively involved in granting survival of bacterial cells in the upper part of the gastrointestinal tract. This selection allowed the organization of four strains for further evaluation of their survival kinetics using TIM.

Three strains survived much better than the fourth one, in all digestive compartments of TIM, possibly because of the presence of urease and heat shock protein functions; survival was also significantly improved when strains were tested as part of fermented milk but not pure cultures.

However, all results obtained by means of in vitro methodology are to be viewed with caution because they do not report the outcome of a real human feeding study.

The overall outcome of these data, suggesting a different ability to survive during gastrointestinal transit between *L. bulgaricus* and *S. thermophilus*, has been supported by a more detailed genomic assessment that will be discussed in the next section.

### 23.4 A GENOMIC VIEW OF YOGURT STARTER CULTURES

The availability of DNA-based techniques provides worthy data on the evolutionary history of yogurt starter cultures as well as on their potential to exert beneficial actions on humans.

*S. thermophilus* is the only species of the genus *Streptococcus* to be deliberately used for food fermentation, but its environmental original niche has not been identified.

This species belongs to the *salivarius* group of the *Viridians* streptococci, which include two other species, *Streptococcus salivarius* and *Streptococcus vestibularis*.

For some years its taxonomic status was as a subspecies of *S. salivarius*, but nowadays it has been recognized as an independent taxonomic unit thanks to DNA–DNA hybridization experiments (Schleifer et al., 1991).

Currently, 30 chromosomal sequences are present in the National Center for Biotechnology Information genome database. Sequence analysis of *S. thermophilus* chromosomes resulted in a median total length of 1.81 Mb, a median protein count of 1731, and a median GC% of 39.09 (further details in Table 23.2).
Comparative genomics of *S. thermophilus* with genomes of streptococcal pathogens (8–13) on the one hand supports its relatedness to pathogenic species but on the other it highlights the lack of determinants for pathogenicity, so reinforcing the safety profiles of this species.

Mechanisms of the evolutionary path that has shaped *S. thermophilus* genomes are loss-of-function as well as horizontal gene transfer events, which have played a major role in causing divergence from genomes of pathogenic streptococci and, in the meantime, achieved its adaptation to a very specific ecological niche such as milk.

As a general rule it could be stated that *S. thermophilus* has a relevant nitrogen metabolism, while sugar catabolism has been reduced.

In addition, while pathogenic streptococci are well known for the high numbers of surface-exposed proteins for promoting adhesion to tissues or to escape the host immune system, *S. thermophilus* has a very low capacity to export proteins, even if, as we will discuss later, certain strains have retained some of this capacity.

A core genome of about 1200 genes has been established, while a little more than 200 genes might have been acquired by horizontal gene transfer.

Of special interests for this chapter are genes related to secreted and/or cell wall-anchored proteins, possibly related to tissue adhesion and interactions with the immune system.

*S. thermophilus* strains appear to have a genomic potential to secrete about 50 proteins in the external medium or as cell wall attached, suggesting that *S. thermophilus* is endowed with the lowest amount of putative secreted proteins known among streptococci; this is a strong indication of a reduced potential to adhere to epithelial tissues.

However, there are specific strains encoding for putative mucus-binding proteins, peptide transporters, and exopolysaccharide (EPS) biosynthetic proteins that have close orthologs in human intestinal microorganisms. This observation could be taken as a preliminary indication that there are some strains that could have retained ancestral features involved in tissue colonization.

There are currently 32 genome assemblies of *L. delbrueckii* reported in international databases, among them 17 belong to the *bulgaricus* subspecies; the main features of *S. thermophilus* and *L. bulgaricus* chromosomes are summarized in Table 23.2.

Contrary to *S. thermophilus*, the original niche of this bacterium is easily predicted, because the other subspecies of *L. delbrueckii* are related to plant and fermenting vegetables, while the milk adaptation pathway of *L. bulgaricus* appears to be similar to that of *S. thermophilus*.

The overall analysis of *L. bulgaricus* genomes strongly suggests that the evolution of *L. bulgaricus* from a plant-associated habitat to the protein and lactose-rich milk environment was achieved through the loss of superfluous functions and also by means of protocooperation with *S. thermophilus* (see later for further details).

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**Table 23.2 Genomes of *Streptococcus thermophilus* and *Lactobacillus bulgaricus***

<table>
<thead>
<tr>
<th></th>
<th>Range of Genome Size (Mb)</th>
<th>GC%</th>
<th>Genes Content</th>
<th>Protein Encoded</th>
<th>Genome Assemblies</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. thermophilus</em></td>
<td>1.60–2.06</td>
<td>38.8–39.2</td>
<td>1707–2381</td>
<td>1476–2193</td>
<td>30</td>
</tr>
<tr>
<td><em>L. bulgaricus</em></td>
<td>1.73–2.06</td>
<td>49.59–50.00</td>
<td>1778–2086</td>
<td>1621–1870</td>
<td>17</td>
</tr>
</tbody>
</table>

*This table has been compiled on the basis of data retrieved from the NCBI genome database in September 2016.*
In *L. bulgaricus* genomes, the large presence of incomplete metabolic pathways and pseudogenes as remnants of ancestral metabolic pathways should be noted. However, peculiar features of *L. bulgaricus* genome support the hypothesis that the genome is still in a phase of rapid evolution, i.e., it harbors a very high number of rRNA and tRNA genes when compared to genome size, suggesting that the *L. bulgaricus* genome has experienced a recent phase of important size reduction.

At the moment, available data on the genomic composition of yogurt starter cultures suggest that these two species are the result of a fast adaptation to the milk environment, and they have lost or inactivated the genetic determinants generally believed to be involved in survival during intestinal transit or crosstalk with the gut epithelia.

However, comparative genomics has opened the door in the search for specific strains still retaining some ancestral genes and able to confer health-related features to consumers; this could be a challenging research area for the future.

### 23.5 COOPERATION BETWEEN THE TWO STARTERS AS ASSESSED BY GENOMICS

*L. bulgaricus* and *S. thermophilus* represent a well-known example of protocooperation when they are cocultivated in milk (Fig. 23.1), and their cooperation in fermented milk has been the focus of several studies (for a review see Sieuwerts, 2016).

![A coculture of *Streptococcus thermophilus* and *Lactobacillus bulgaricus* as seen by means of scanning electron microscopy.](image-url)
Analysis of this cooperation is beyond the scope of this chapter, but it appears worthwhile to point out that bioinformatic analysis performed on available genomes of both species has shown a large presence of horizontally transferred genes in both *L. bulgaricus* and *S. thermophilus*.

Among them it is relevant to note that *S. thermophilus* appeared to be the donor to *L. bulgaricus* (*Liu et al., 2009*) of EPS biosynthesis genes; if these EPSs could have a role in the adhesion to intestinal tissues or other health-related features. It is a tempting hypothesis, but it still needs to be investigated.

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### 23.6 YOGURT BACTERIA AND HEALTH: THE BEGINNING

As mentioned before, the relevance of selection of strains able to survive in the human gut to exert a beneficial action was present in the original work of Metchnikoff. In *Chapter 5* (Part III) of his famous book *The Prolongation of Life*, Metchnikoff took into consideration a range of fermented dairy products, and then focused his attention on a bacterium already used in experiments with human volunteers and isolated from Bulgarian yogurts.

Metchnikoff provided a description of this “Bulgarian Bacillus” (sic) as a very fast milk coagulating one, able to produce 25 g/L of lactic acid when grown in milk.

This is a quite puzzling observation, because strains currently classified as “bulgaricus” are not generally believed to produce this high level of acid, which is typical for *L. helveticus* strains (*Kandler and Weiss, 1986*). It may be worthwhile pointing out that in international culture collections, there is only one strain described as “one of the original Metchnikoff strains (ATCC 521=CIP 76. 19=JCM 1003=NCFB 87=NCIB 2889=DSM 11445).”

Curiously, this strain was deposited as *Lactobacillus jugurti*, a species nowadays recognized as a biotype of *L. helveticus* (*Dellaglio et al., 1973*).

Metchnikoff founded a dairy company called “Produits à la lactobacilline” located in Paris on Rue Denfert-Rochereau; the company commercialized a fermented milk called “Lactobacilline” prepared with cultures of lactic acid bacteria without “microbes present…in acidified milk such as kephir, yahourt, varenets, etc.”

The expected health benefit was an action against “putrefactive” pathogenic bacteria, a beneficial action focused on gastrointestinal health.

Since that time, a continuous flow of research has focused on the action of yogurt on health; one recent review (*Glanville et al., 2015*) has identified 213 studies of yogurt and health, covering areas such as bone health, weight management, metabolic health, cardiovascular health, gastrointestinal health, cancer, diabetes, Parkinson’s disease risk, all-cause mortality, skin complaints, respiratory complaints, joint pain/function, as well as eight studies reporting a number of different outcomes. The authors concluded that there is enough scientific knowledge to conduct a meta-analysis, in addition to the two already available and focused on the gastrointestinal beneficial effects of yogurt (see below).

In the following section an evaluation of some of these reported outcomes has been presented, with special attention paid to those possibly more related to the action or presence of bacterial starters than to nutritional-related health outcomes.
23.7 YOGURT AND GUT HEALTH

The beneficial effects of yogurt containing viable cultures have been taken into consideration by two recent metaanalyses, in which intervention studies based on yogurt as defined by the Codex Alimentarius standard for fermented milk (CODEX STAN 243-2003), at any dosage scheme and duration of delivery, were reviewed.

In the first one (Patro-Gołąb et al., 2015a), the focus was on yogurt for treating acute gastroenteritis in children.

Four randomized controlled trials (RCTs) for a total of 448 subjects, with an age range between 3 and 48 months, all performed in a hospital setting, were evaluated. Compared with placebo/no intervention, yogurt consumption had no significant effect on stool volume as well as on the duration of diarrhea and stool frequency. However, the duration of hospitalization was shorter in children who received yogurt, even if the difference was of a borderline significance. The only clearly positive outcome was that the total weight gain increased for those who consumed yogurt.

The latter metaanalysis (Patro-Gołąb et al., 2015b) was focused on antibiotic-associated diarrhea. Even if it were possible to include only two RCTs, which the authors classified as low in methodological quality, the authors concluded that yogurt consumption reduced the risk for diarrhea, at least in the fixed model effect, compared with the control groups with no intervention. However, significant heterogeneity between the trials was detected and any significant reduction in the risk for diarrhea was lost if the random effects model of assessment was used, pointing out the scarcity of available data.

As a conclusion to this section, it is surprising to note that the health effect originally attributed to yogurt, the maintenance of intestinal health, has been addressed by a small number (six as reviewed by the two cited metaanalyses) of clinical studies.

23.7.1 LACTOSE MALDIGESTION, CALCIUM INTAKE, AND YOGURT

However, the most recognized health effect, from both scientific academia and regulatory bodies, delivered by yogurt and its starter cultures is the reduction of symptoms related to lactose maldigestion, which is related to the presence of viable cells at the time of ingestion but not during intestinal transit (Guarner et al., 2005).

This effect is shared by all yogurt starter cultures, because of the presence of the lactose-hydrolyzing enzyme in all strains of the used species of lactic acid bacteria; this species-related trait has been recognized also at the regulatory level by FAO/WHO (2001, 2002) and EFSA (2010) and it does not require survival and reproduction of the bacterial cells during intestinal transit.

It appears interesting to review the assessment made by the European Food Safety Authority (EFSA) to approve the claim, according to Regulation EC 1924/2006, that: “Live yoghurt cultures in yoghurt improve digestion of lactose in yoghurt in individuals with lactose maldigestion.”

Before providing details on the evaluation process, there is a need to point out that the claim asks for bacterial viability and that it is related to bacteria contained in yogurt and not to food supplements or the delivery vehicles of the bacterial cultures.

The EFSA panel in charge of the evaluation of the action of yogurt on the improvement of lactose digestion took into consideration 14 human intervention studies.

All of them were performed using the yogurt cultures, L. delbrueckii ssp. bulgaricus and S. thermophilus, according to Codex specifications.
In 10 out of 14 studies, lactose digestion has been investigated for a single dose of yogurt, whereas in the remaining four, lactose digestion was studied after prolonged ingestion of fresh or heat-treated yogurt.

Comparison of results obtained by using viable/unviable yogurt starter cultures clearly supports the need of cells viable at the time of oral ingestion, which is possibly the best way to guarantee the survival of the bacterial hydrolytic enzyme during gastric transit, and is also supported by the buffering action of yogurt.

The slow transit time throughout the small intestine is possibly related to the release of lactase from lysing cells in an amount able to digest the residual lactose, avoiding action that may trouble lactose maldigesters.

This action is possible only for the subgroup of lactic acid bacteria in which lactose is digested by means of an ATP-based lactose transport system, such as yogurt bacteria, whose lactase does not require any previous phosphorylation of the sugar substrate; the latter subgroup, which is endowed with a phosphoenolpyruvate:carbohydrate phosphotransferase system lactose utilization system, does require actively metabolizing cells to digest lactose.

Because the property is related to a metabolic pathway shared by all strain members of the two species, EFSA did not restrict specific strains but allowed the use of the claim to all yogurt starter cultures composed of \( S. \) \( \text{thermophilus} \) and \( L. \) \( \text{bulgaricus} \). This evaluation is also present in one FAO/WHO document (2002) and also has scientific consensus (Guenner et al., 2005).

An additional health effect, related to the beneficial action for lactose maldigesters, is the role played by yogurt in improving calcium availability for this specific population group.

A report (Brown-Riggs, 2015) has stated that the National Medical Association and the National Hispanic Medical Association of the United States issued a joint consensus statement recommending African-Americans to consume three to four servings of low-fat dairy every day; they also suggested that cultured dairy products could play an important role in addressing these recommendations because of the presence of lactase-producing cultures, and that yogurt is often a more easily digestible alternative to milk and thus more palatable to people who experience symptoms of lactose intolerance.

This official position is the most recent of a long list of similar guidelines or position papers, such as the “Commentary from the Belgian Bone Club and the European Society for Clinical and Economic Aspects of Osteoporosis, Osteoarthritis and Musculoskeletal Diseases” (Rozenberg et al., 2016), which clearly supports the consumption of yogurt: “Lactose intolerant individuals may not need to completely eliminate dairy products from their diet, as both yogurt and hard cheese are well tolerated.” It is interesting to note that for calcium absorption, it appears that it is necessary to have viable yogurt starter cultures.

In a study reported by Parra et al. (2007), fresh and pasteurized yogurts were compared to measure the calcium intake by lactose-intolerant subjects.

Assimilation was measured by means of the stable isotope \( ^{43}\text{Ca} \) in 40 volunteers (age 32±7 years); 20 of them were suffering from moderate lactose intolerance.

Volunteers consumed a test meal consisting of \( ^{43}\text{Ca} \)-labeled fresh or pasteurized yogurt and the calcium status was assessed in 24-h urine before and after the test meal by using isotopic rate mass spectrometry.

In both lactose tolerant and intolerant subgroups of volunteers, the consumption of fresh yogurt containing viable cells provided an outcome of statistically higher circulating calcium levels as compared with results obtained when the same measurements were carried out in volunteers fed with
pasteurized yogurt. The authors concluded that comparison of calcium utilization from two different dairy sources revealed a higher acute calcium assimilation from fresh as compared to pasteurized yogurt in both lactose digesting and maldigesting subjects.

### 23.7.2 STRAIN-RELATED BENEFICIAL ACTIONS

In the previous sections, it was clear that, while the positive action exerted toward lactose maldigestion is shared by all members of the two species, behavior of yogurt cultures in the gut may vary from strain to strain, and, possibly, beneficial actions.

This observation is quite puzzling and scientifically challenging; an extensive search in the existing literature to verify if there is support for strain specificity for certain health-enhancing actions possibly exerted by different strains of yogurt cultures, resulted in a positive outcome. There are indeed some papers in which a strain-related beneficial action has been demonstrated.

Two major outcomes were the results of this search: one was related to the action toward the immune system exerted by an *L. bulgaricus* strain and the other was related to the ability of selected yogurt cultures to enrich the vitamin content of yogurt.

The action of *L. bulgaricus* on the immune system is not particularly new in the area of yogurt research, but the novelty of a series of papers published by a Japanese group (Kano et al., 2002a,b, 2013; Makino et al., 2006, 2010, 2016; Nagai et al., 2011) showed that they were able to reveal in vitro and in the animal model as well as in one human study of the immune modulation exerted by a specific strain of *L. bulgaricus*, called OLL1073R-1. This action was also identified in a capsular polysaccharide, the bacterial component in charge of this action.

The *L. bulgaricus* strain OLL1073R-1 was selected among a group of 139 *L. bulgaricus* strains as the most robust producer of EPS. Biochemical characterization of this capsular component showed that its sugar moiety is formed by D-glucose and D-galactose. It was assumed that this capsular polysaccharide could exert an immunostimulatory effect and a number of animal model studies showed stimulation of mouse splenocytes and a significant increase of interferon-gamma production (Nagai et al., 2011; Kano et al., 2013) with an overall marked effect on the immune system of mice.

Moreover, when orally administered to mice, the purified polysaccharide was shown to augment natural killer cell activity, suggesting a clear link between this outer envelope of bacterial cells and the action on the immune system.

The next step was to show once again in the animal model the beneficial effects of this strain on the development of autoimmune disease. Kano et al. (2002a), using collagen-induced arthritis (CIA) as a model of some types of rheumatoid arthritis, showed that oral intake of milk fermented with *L. bulgaricus* OLL1073R-1 was found to reduce the insurgence and development of CIA in mice, compared with a control group fed a control feed fermented with a different strain of *L. bulgaricus*.

The strain showed its peculiar properties even when used not only as a pure culture but also as a starter. Yogurt produced using *L. bulgaricus* OLL1073R-1 and fed to mice showed a similar level of immunomodulation as the purified polysaccharide, but this action was not present in yogurt fermented with a different strain of *L. bulgaricus* (Makino et al., 2016). This also supports the concept of strain specificity.

These results suggested that yogurt fermented with *L. bulgaricus* OLL1073R-1, containing immunostimulative EPS, could have an immuno-modulatory effect on the human body.
However, efficacy for human health is still to be demonstrated in humans; for this strain, only one clinical trial in humans was performed, showing that this strain is able to reduce the incidence of common cold in elderly when administered daily with yogurt.

In two independent studies, Makino et al. (2010) recruited 57 (median age 74.5 years) and 85 healthy elderly individuals (median age 67.7 years). In each study, there was a treatment group (fed with 90 g of yogurt per day) as well as a placebo group.

A statistical analysis of the results of these two independent studies showed a marked reduction of the insurgence of the common cold among the yogurt-consuming subjects (about 2.6 times lower with an odds ratio of 0.39 and \( P = .019 \)).

As regards the measured biomarkers, in the yogurt group the increase of natural killer cell activity was significantly higher than in the milk group (\( P = .028 \)), and also the quality of life score for the “eye/nose/throat” system was significantly higher in the yogurt group than in the milk group. The authors’ conclusions were that “consumption of yogurt fermented with \( L. \) bulgaricus OLL1073R-1 augmented natural killer cell activity and reduced the risk of catching the common cold in elderly individuals.”

Less is known about the strain-specific, health-related properties of strains belonging to the \( S. \) thermophilus species; however, available data for experiments performed in mice and rats (Kano et al., 2002a,b, 2013; Nagai et al., 2011) strongly suggested that there is the possibility that EPS-producing strains could exert an immuno-modulatory action.

An additional example of a beneficial action, not related to lactose digestion, exerted by yogurt cultures is the improvement of the vitamin B profile in adults, with special attention paid to young healthy women (Fabian et al., 2008).

A group of nutritionists based in Vienna, Austria, conducted a study in which volunteers consumed 100 g/day of probiotic (\( n = 17 \)) or conventional yogurt (\( n = 16 \)) for 2 weeks (\( T1–T2 \)) and 200 g/day for a further 2 weeks (\( T2–T3 \)). Plasma and urine concentrations of thiamine, riboflavin, and pyridoxine were determined; the main outcome of this study was that the plasma levels of vitamin B1 increased in both groups (\( P < .01 \)) with no differences between probiotic and plain yogurt.

The authors also added an interesting observation: “The diminished plasma flavin adenine dinucleotide and increased flavin mononucleotide concentrations, observed during the period of daily yogurt consumption in both groups, may be the result of enhanced immune function and an oxidant/antioxidant imbalance, caused by the daily intake of lactic acid bacteria.”

Moreover, the authors observed that the long-term status parameters of all three investigated vitamins (B1, B2, and B6) was not altered during the trial and that changes in plasma concentrations appear more likely due to the result of standard yogurt feeding, rather than of the specific intake of probiotic bacteria.

It appears highly possible that vitamin production could be strain related; genomic studies will be relevant to select the most actively producing vitamin cultures. It is then possible to conclude that a new research line is open for scientists to assess and exploit the strain-specific beneficial properties of traditional yogurt starter cultures.

A strain difference was also reported regarding a potentially negative trait of \( S. \) thermophilus, that is histamine production (Calles-Enríquez et al., 2010; Gezginc et al., 2013).

After elucidation of the genetic background of this metabolic activity (Calles-Enríquez et al., 2010), formation of 12 different biogenic amines by 58 isolates of \( S. \) thermophilus (Gezginc et al., 2013) from homemade natural yogurt was investigated in histidine decarboxylase broth (HDB) and lysine decarboxylase broth (LDB).
All \textit{S. thermophilus} isolates had an ability to produce 12 different biogenic amines in HDB and LDB. Most of the \textit{S. thermophilus} isolates formed low amounts of histamine (1–50 mg/L) from histidine. Apart from one isolate, \textit{S. thermophilus} produced tyramine at low (47 isolates) and medium (10 isolates) levels. The amount of each specific biogenic amine produced by \textit{S. thermophilus} was generally lower than 100 mg L(−1). Also the presence of the \textit{hdcA} gene was investigated using the PCR technique and the relation between gene and histamine production was conducted in \textit{S. thermophilus} isolates. This study showed that most of the \textit{S. thermophilus} isolates have the ability to form biogenic amines, especially histamine and tyramine; this is an important consideration when selecting strains as starter cultures.

23.8 CONCLUSIONS

A wealth of literature clearly supports that the fermentation of milk by \textit{L. bulgaricus} and \textit{S. thermophilus} produces, as a final outcome, a food product endowed with beneficial properties that are more than expected by the simple sum of the ingredients. The case of lactose maldigestion is a good example of this than addictive effects of the two bacterial components and the density of the fermented milk.

While positive effects related to the gastrointestinal environment have reached an intensity of research to be analyzed through metaanalysis, the beneficial effect on other sites of the human body are still to be investigated in depth. Yogurt with its bacterial culture is therefore a traditional food with a long history but also a long future.

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24.1 INTRODUCTION

About 2000 years ago, Hippocrates emphasized “Let food be your medicine and medicine be your food.” Currently, there is an increased global interest in “nutraceuticals” as they play a major role in health enhancement. Accordingly, “nutraceutical” is any substance that may be a food or part of a food and provides medical or health benefits, encompassing, preventing, and treating of diseases. Such products may range from isolated nutrients, dietary supplements, and diets to genetically engineered “designer” foods, herbal products, and processed foods (Rajasekaran et al., 2008). Nutraceuticals and functional foods have received considerable interest because of their presumed safety and potential nutritional and therapeutic effects. The nutraceutical and functional food industry is in a unique position to capitalize on consumers’ interest. When functional food aids in the prevention and/or treatment of disease(s)/disorder(s) other than deficiency conditions such as anemia, it is called a “nutraceutical” (Kalra, 2003).

Milk forms a rich source of such nutraceuticals; in particular its protein fraction and its derivatives are known to encompass many health-promoting effects. Milk protein-derived biologically active peptides are currently the subject of intensive research. These bioactive peptides are a sequence of amino acids that are encrypted within the primary structure of milk proteins, requiring proteolysis for their release from the precursor. These are released during enzymatic digestion in vitro or in vivo (Korhonen and Pihlanto, 2003). Biological activities associated with such peptides include immuno-modulatory, antibacterial, antihypertensive, antioxidative, and opioid-like properties. Proteolytic activation of a bioactive sequence by lactic acid bacteria (LAB) has the great advantage of using food-grade microorganisms to enrich foods with bioactive substances. A proteolytic system of LAB has extracellularly located serine proteinases, a transport system specific for di-, tri-, and oligopeptides, and a multitude of intracellular peptidases (Kunji et al., 1996). Thus LAB could potentially generate a large variety of peptides, including bioactive sequences based on the specificity of proteolysis.

Yogurt is a coagulated milk product obtained from lactic acid fermentation by the action of Lactobacillus delbreuckii ssp. bulgaricus and Streptococcus thermophilus (Anonymous, 2009). Yogurt is traditionally considered as a healthy food and the functionality of yogurt is further enhanced by the release of bioactive peptides during lactic fermentation (Shah, 2007).
24.2 YOGURT

Yogurt means a coagulated product obtained from pasteurized, boiled, or concentrated milk, pasteurized skimmed milk and/or pasteurized cream, or a mixture of two or more of these products by lactic acid fermentation through the action of \textit{L. delbrueckii} ssp. \textit{bulgaricus} and \textit{S. thermophilus}. It may also contain cultures of \textit{Bifidobacterium bifidus}, \textit{Lactobacillus acidophilus}, and other cultures of suitable lactic acid-producing bacteria. High-temperature pasteurization of the yogurt mix is employed to obtain a smooth and firm body. Nonfat dry milk or stabilizers may also be added to increase the water-holding capacity and therefore improve its body. The latter is particularly applicable to low-fat products.

Several different types of yogurt are commercially available. These include plain (no added flavors), flavored, liquid, carbonated, and low lactose. The flavored yogurts include the sundae style in which fruit purée is layered at the bottom of the cup and is mixed with the yogurt before consumption. The other type is Swiss style, in which plain yogurt is gently blended with fruit purée before packaging. Such yogurts require high levels of solids and stabilizer to obtain the desired high viscosity. Liquid yogurts are popular in Europe, Canada, and Japan, and differ from gel-type yogurts in that they are in a homogeneous, pourable state.

No whey separation should occur during storage. The manufacture of yogurt involves several key steps: standardization of mix, homogenization, heat treatment, cooling to incubation temperature, inoculation with yogurt cultures, incubation, cooling, and packaging (\textit{Mistry, 2001}).

24.3 FERMENTATION

24.3.1 MICROBIOLOGICAL ASPECT

Codex regulations define yogurt as the product obtained by fermenting milk with a culture that includes \textit{L. delbrueckii} ssp. \textit{bulgaricus} and \textit{S. thermophilus}. They are thermophilic organisms and grow best at approximately 45°C but not above 50°C. They are typically added in a 1:1 ratio and function symbiotically to produce typical yogurt characteristics. Either culture independently is unable to produce the ideal balance of acid and flavor. \textit{S. thermophilus} initiates lactic acid production and lowers the oxygen level, which stimulates growth of \textit{L. delbrueckii} ssp. \textit{bulgaricus}. The pH is lowered to approximately 5 by the cocci and then to less than 4 by the rods (\textit{Mistry, 2001}). The interaction depends on the fact that \textit{S. thermophilus} grows more rapidly than \textit{L. delbrueckii} ssp. \textit{bulgaricus} in milk, and ferments lactose homofermentatively to give L (+) lactic acid as a principal product. In addition, carbon dioxide is liberated by the breakdown of urea in the milk by urease and, usually, formic acid (up to 40 \(\mu\)g/mL); all three metabolites stimulate the growth of \textit{L. delbrueckii} ssp. \textit{bulgaricus} (\textit{Robinson, 2000}). Some free amino acids occur naturally in the milk or are released during heat treatment. However, some amino acids, such as glutamic acid, histidine, cysteine, methionine, valine, or leucine, are not present at levels sufficient to support extensive growth of the culture. Nevertheless, \textit{L. delbrueckii} ssp. \textit{bulgaricus} can hydrolyze casein—especially \(\beta\)-casein—by means of a cell wall protease to release polypeptide and, by further enzymatic activity, free amino acids as well. In addition, \textit{S. thermophilus} can readily hydrolyze peptides so that, again, the free amino acids that are essential for further development of both species become available. \textit{S. thermophilus} is more sensitive to acid than \textit{L. delbrueckii} ssp. \textit{bulgaricus}; hence, during extended storage of yogurt, the former are likely to be injured by the acid and gradually die off. Therefore, although the initial ratio of rods to cocci may be 1:1, this ratio may change in favor of lactobacilli during storage of the yogurt (\textit{Robinson and Itsaranuwat, 2006}).
24.3.2 BIOCHEMICAL ASPECT: PROTEOLYSIS

Although, yogurt and bio starter cultures are considered to be weakly proteolytic, *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* may cause a significant degree of proteolysis during fermentation and this activity may be important for the following reasons (Tamime and Robinson, 1999):

1. The enzymatic hydrolysis of milk proteins results in the liberation of peptides of varying sizes and free amino acids, and these possible changes may be involved during the formation of the gel and can affect the physical structure of yogurt.
2. The liberation of amino acids into the milk is essential for the growth of *S. thermophilus*.
3. Although amino acids and peptides may not contribute directly toward the flavor of yogurt, they do act as precursors for the multitude of reactions that produce flavor compounds.
4. Therapeutic potential may be increased by the release of so-called functional peptides or bioactive peptides.

The range of products released by proteolysis is dependent on two main factors: first, the components of the milk protein fraction and second, the types of proteolytic enzyme that the yogurt and the bioorganisms may possess.

24.3.3 CONSTITUENT COMPOUNDS OF MILK PROTEIN

The major protein fractions in bovine milk consist of caseins (α_S1, α_S2, β, and κ) and whey proteins which include immunoglobulins, α-lactalbumin, β-lactoglobulin, bovine serum albumin, immunoglobulin, lactoferrin, as well as proteose peptone fractions and transferrin. The preheat treatment of milk before inoculation leads to the partial denaturation of milk proteins and facilitates the action of proteolytic enzymes during fermentation.

24.3.4 PROTEOLYTIC ENZYMES

LAB have a limited capacity to synthesize amino acids and are therefore dependent on the use of exogenous nitrogen sources for optimal growth. Because milk contains only small amounts of amino acids and short peptides, LAB depend on a complex proteolytic system to obtain essential amino acids from caseins during growth in milk. This specialized proteolytic system consists of a cell envelope-associated proteinase (CEP), transport systems to allow uptake of the resulting peptides, and several intracellular peptidases, which degrade peptides to amino acids. The CEP is the key enzyme of this system because it is involved in the first step of casein degradation. In addition to its vital role for bacterial growth in milk, CEP may release bioactive health-beneficial peptides during milk fermentation. One approach to improve bioactive peptide production is to enhance CEP activity involved in peptide generation, which requires characterization of CEP.

Proteolytic activity of *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* indicates that both organisms possess different exopeptidases and peptidases. *S. thermophilus* is considered to have more exopeptidase activity than *L. delbrueckii* ssp. *bulgaricus* and only limited endopeptidase activity. The characteristic properties of endopeptidase from *L. delbrueckii* ssp. *bulgaricus* are that the enzyme is a zinc-dependent monomer of ~70kDa, which degrades intact casein with a significant preference for β-casein. The caseinolytic activity increases as the pH is lowered (<5.0), which suggests that the enzyme might be involved during the later stages of the fermentation period. These endopeptidases...
hydrolyze the casein to yield polypeptides, which in turn are broken down by the exopeptidases of *S. thermophilus* with the liberation of amino acids (Tamime and Robinson, 1999).

Following the hydrolysis of casein, the peptides are broken down further by the exopeptidases of *S. thermophilus* for the release of amino acids. Three main categories of exopeptidases are produced by LAB: (1) aminopeptidase N (PepN) is a monomeric intracellular metallopeptidase with molecular weight ∼95 kDa present in *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus*, which is capable of cleaving the N-terminal amino acids; (2) aminopeptidase C (PepC) is a thiol peptidase similar to PepN with a molecular weight ∼50 kDa and is capable of removing a broad range of N-terminal residues of peptides; and (3) X-prolyl-dipeptidyl-aminopeptidase (PepX) is a serine protease present in *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus*, which has the ability to release dipeptide from oligopeptide even when proline is in the penultimate position (Tamime and Robinson, 1999).

The rate of proteolysis is very high during the log phase and decreases gradually during storage or after the stationary phase. The ratio of *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* in the starter culture and during the storage period can affect the level of amino acids in yogurt, e.g., 70 mg/100 g amino acid is liberated at a ratio of 1:1 after 1 day, which decreases to 50 mg/100 g after 2 days and 41 mg/100 g after 5 days. However, the acidity of these yogurts remains rather high, i.e., 1.9 g/100 g lactic acid for the 1:1 ratio. The high level of liberated amino acids in the product might be associated with the proteolytic activity of *L. delbrueckii* ssp. *bulgaricus*, which becomes the predominant organism in such an acidic environment.

### 24.3.5 PRODUCTS OF PROTEOLYSIS

The level of nitrogenous compound in yogurt is varied as compared to that with milk because of the proteolytic activity of *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus* during the fermentation period and, to a lesser degree, during cold storage of the product. The variation is mainly caused by the release of soluble nitrogenous compounds, which include free amino acids and peptides from milk proteins. Because of the complexity and multiplicity of the proteolytic system, LAB would produce a greater diversity of peptides from milk proteins compared to digestive proteases. Strains of LAB have been shown to cleave peptide bonds such as the imino group of prolines and the “strategic regions” of casein containing overlapping peptide sequences with various biological activities, which are protected from breakdown by digestive proteases. The choice of strains influences the release of effective bioactive peptides. Nonetheless, the complexity and multiplicity of the proteolytic system in LAB may also be a disadvantage if not adequately managed. Therefore strains should not be too proteolytic otherwise the product will deteriorate and must have the right specificity to give high concentrations of active peptides. Proteolysis should be managed so that the ratio of soluble nitrogen to total nitrogen (\(\frac{N_{\text{soluble}}}{N_{\text{total}}}\)) remains between 12% and 24% (Rizzello et al., 2005). The concentration of bioactive peptides appears to rely on a balance between their formation and further breakdown into inactive peptides and amino acids, which in turn depends on storage time and conditions.

### 24.3.6 BIOACTIVE PEPTIDES FROM YOGURT

The value of proteins as an essential source of amino acids is well documented, but it has been recognized that dietary proteins exert many other functionalities in vivo by means of biologically active peptides. Such peptides are inactive within the sequence of the parent protein and can be released by
digestive enzymes during gastrointestinal transit or by fermentation or ripening during food processing. Bioactive peptides have been defined as specific protein fragments that have a positive impact on body functions or conditions and may ultimately influence health (Kitts and Weiler, 2003). At present, milk proteins are considered the most important source of bioactive peptides. Upon oral administration, bioactive peptides may affect the major body systems—namely, the cardiovascular, digestive, endocrine, immune, and nervous systems. For this reason, the potential of distinct dietary peptide sequences to promote human health by reducing the risk of chronic diseases or boosting natural immune protection has aroused increasing scientific and commercial interest over the past decade (Hartmann and Meisel, 2007). The activity of these peptides is based on their inherent amino acid composition and sequence. The size of active sequences may vary from 2 to 20 amino acid residues, and many peptides are known to reveal multifunctional properties. Proteolytic activation of bioactive sequence by LAB has the great advantage of using food-grade microorganisms to enrich foods with bioactive substances. Bioactive peptides from fermented dairy products can be divided into different categories based on their physiological effect on the body or the protein from which they have been derived; antihypertensive, antioxidant, antithrombotic, opioid, casein phosphopeptides (CPPs), antimicrobial, cytomodulatory, immuno-modulatory, and miscellaneous peptides (Table 24.1). These functions relate to general health conditions or a reduced risk of certain chronic diseases (for reviews, see Nongonierma and FitzGerald, 2015a,b; Mills et al., 2011; Korhonen and Pihlanto, 2006; Korhonen, 2009).

24.3.7 ANTIHYPERTENSIVE PEPTIDES

Cardiovascular diseases (CVDs) are the major cause of death in humans globally (WHO, 2013). High blood pressure (BP) is a modifiable risk factor for CVD. The angiotensin I-converting enzyme (ACE, peptidyl dipeptide hydrolase, EC 3.4.15.1) has been associated with the renin–angiotensin system, which regulates peripheral BP. Inhibition of this enzyme can exert an antihypertensive effect. A great number of ACE-inhibitory peptides have been isolated from fermented milk products and at present they are the most studied group of bioactive peptides. Apart from ACE inhibition, milk peptides may also exert antihypertensive effects through other mechanisms, such as inhibition of the release of endothelin-1 by endothelial cells (Maes et al., 2004), stimulation of bradykinin activity (Perpetuo et al., 2003), enhancement of endothelium-derived nitric oxide production (Sipola et al., 2002), and enhancement of the vasodilatory action of binding to opiate receptors (Nurminen et al., 2000).

Many studies have been conducted for the antihypertensive effect of fermented milk products, but only a few have been reported for yogurt. Milk fermentation using LAB or their proteinases has been described as a strategy to release ACE-inhibitory peptides from milk proteins, especially caseins (Hayes et al., 2007). Donkor et al. (2007) evaluated ACE-inhibitory activity in yogurt containing additional probiotic strains during refrigerated storage. Yogurt was prepared using either a sole yogurt culture including L. delbrueckii ssp. bulgaricus Lb1466 and S. thermophilus St1342, or with L. acidophilus L10, Lactobacillus casei L26, or Bifidobacterium lactis B94 besides the yogurt culture. All probiotic yogurts showed greater ACE-inhibitory activity during the initial stage of storage (first 3 weeks) versus the control; however, activity decreased afterward and IC_{50} ranged from 27.79 to 103.30 μg/mL in whey fractions. ACE-inhibitory peptides Val-Pro-Pro (Clare et al., 2003) and Ile-Pro-Pro (Chabance et al., 1995) derived from casein following fermentation with the strains Lactobacillus helveticus and Saccharomyces cerevisiae, respectively, are found in the commercial sour milk product Calpis (Calpis, Co. Ltd., Tokyo). In vivo studies with spontaneously hypertensive rats (SHR) represent a useful animal
### Table 24.1 Bioactive Peptides Identified From Yogurt

<table>
<thead>
<tr>
<th>SI. No.</th>
<th>Peptide Sequence</th>
<th>Protein Fragment</th>
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<tr>
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<td>PLW</td>
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<td>5</td>
<td>FVAP</td>
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<td>TTmplw</td>
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### 2. Antioxidant Peptides

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<td>QQPVLGPVRGPPII</td>
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<td>7</td>
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<td>ARHPHPLSF</td>
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### 3. Opioid Peptides

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Table 24.1 Bioactive Peptides Identified From Yogurt—cont’d

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<td>κ-CN (18–29)</td>
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<td>5. Mineral-Binding Peptides</td>
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<tr>
<td>1</td>
<td>VVRNAN</td>
<td>α-S2CN (43–48)</td>
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ACE, Angiotensin I-converting enzyme.


model to evaluate the antihypertensive effect of fermented milk. The antihypertensive effect of Calpis was shown to yield a decrease in systolic BP of 17.7 mmHg following administration of 5 mL/kg body weight of Calpis sour milk drink over an 8-h period in SHR (Yamamoto et al., 1994). Likewise, a significant reduction in BP was observed in mildly hypertensive patients following oral consumption of 95 mL Calpis over an 8-week period (Hata et al., 1996).

To exert an antihypertensive effect after oral ingestion, active peptides must be absorbed in an intact form from the intestine and further be resistant to degradation by plasma peptidases to reach the target sites. In fact, it has been demonstrated using monolayer-cultured human intestinal Caco-2 cells that the ACE-inhibitory tripeptide VPP can be transported intact through the cell layer via paracellular and transcellular routes, although a significant amount of the peptide is hydrolyzed to amino acids by intracellular peptidases (Satake et al., 2002). It is also known that proline-containing peptides are generally resistant to degradation by digestive enzymes. Masuda et al. (1996) detected two ACE-inhibitory tripeptides (VPP and IPP) in the abdominal aorta of SHR after oral administration of sour milk containing these tripeptides. Foltz et al. (2007) demonstrated that the tripeptide Ile-Pro-Pro selectively escapes from intestinal degradation and reaches the circulation undegraded. It was also demonstrated that Val-Pro-Pro and Ile-Pro-Pro have the potential to inhibit ACE in a very similar fashion to the current synthetic ACE inhibitors captopril, enalaprilat, and lisinopril by hydrogen bonding with similar residues in the ACE catalytic site (Pina and Roque, 2008). A placebo-controlled study tested the effect of “Dahi,” a fermented milk containing the ACE-inhibitory peptide Ser-Lys-Val-Tyr-Pro (SKVYP), on hypertensive subjects (Ashar and Chand, 2004). The product was produced by fermentation of milk with L. delbrueckii ssp. bulgaricus, S. thermophilus, and Lactococcus lactis ssp. lactis biovar. diacetylactis. The subjects received either 100 mL of the test product or the placebo product for 4 weeks. In the test group, a significant decline in systolic BP was recorded after 2 and 4 weeks from the start-up of the trial. No significant change in BP was noticed in the placebo group during the intervention period. The placebo product was prepared using the same starters as the test product, but these strains did not produce the above ACE-inhibitory peptide.
24.3.8 ANTIOXIDANT PEPTIDES

Oxidative stress, the increased production of reactive oxygen species and reactive nitrogen species, combined with overtaking endogenous antioxidant defense mechanisms is a significant causative factor for the initiation or progression of several lifestyle-mediated diseases (Moskovitz et al., 2002). Dietary consumption of antioxidants appears to benefit endogenous antioxidant defense strategies in the fight against oxidative stress (Fang et al., 2002). Studies have shown that peptides having antioxidant properties can be released from milk proteins (Pihlanto, 2006).

The exact mechanism underlying the antioxidant activity of peptides has not fully been understood, yet various studies have shown that these are inhibitors of lipid peroxidation, scavengers of free radicals, and chelators of transition metal ions (Moure et al., 2006; Qian et al., 2008; Rajapakse et al., 2005). In addition, it has been reported that antioxidative peptides exert their effect by intracellular conversion of cysteine to glutathione, a potent intracellular antioxidant, which keeps cells safe from damage by reactive oxygen species through the induction of genes (Marshall, 2004). Antioxidative properties of the peptides are more related to their composition, structure, and hydrophobicity (Chen et al., 1998). Tyr, Trp, Met, Lys, Cys, and His are examples of amino acids that cause antioxidant activity (Wang and Mejia, 2005). As a contribution to antioxidant potency, amino acids with aromatic residues can donate protons to electron-deficient radicals. This property improves the radical-scavenging properties of the amino acid residues. It is proposed that the antioxidative activity of His-containing peptides is in relation with the hydrogen-donating, lipid peroxyl radical-trapping, and/or metal ion-chelating ability of the imidazole group (Rajapakse et al., 2005). On the other hand, the sulfhydryl group in cysteine has an independently crucial antioxidant action because of its direct interaction with radicals (Qian et al., 2008). In addition to the presence of particular amino acids, their correct positioning in the peptide sequence plays an important role in the antioxidant activity of peptides (Rajapakse et al., 2005). Saito et al. (2003) have reported that any change in the arrangements of amino acid sequence in tripeptides resulted in different antioxidant activities. Peptide linkage and/or specific structural features of the peptides have been claimed to influence antioxidant capacity. In contrast to these findings, other results indicated that the peptide bond or its structural conformation can reduce the antioxidant activity of the constituent amino acids. Therefore apparently peptide conformation behaves as a double-edged sword; i.e., it is capable of showing both synergistic and antagonistic effects, as far as the antioxidant activity of free amino acids is concerned (Hernandez-Ledesma et al., 2005). Moreover, it has been stated that the configuration of peptides can also affect antioxidant activity. Chen et al. (1996) found that substitution of L-His by D-His in an antioxidative peptide leads to reduction of the activity. They concluded that the correct positioning of the imidazole group is the key factor influencing antioxidant activity. Finally, it has been postulated that the overall antioxidative activity must be ascribed to the integrative effects of these actions rather than to the individual actions of peptides (Chen et al., 1998).

Many studies have been carried out to evaluate the antioxidant property of fermented dairy products. Antioxidant activity depends on processing conditions such as preheat treatment, incubation period of milk, and type of strain used for fermentation. Thermal treatment of milk before inoculation leads to protein unfolding and exposure of the thiol group potentially acting as a hydrogen donor (Tong et al., 2000; Virtanen et al., 2007). The effect of a prebiotic (fructooligosaccharide; FOS) or a synbiotic component (prebiotic and probiotic) on the proteolysis and antioxidant properties of yogurt has been studied by Madhu et al. (2012) and the results showed that 2,2-diphenyl-1-picrylhydrazyl (DPPH)-radical-scavenging activity and ferric-reducing power in synbiotic yogurt containing Lactobacillus
**plantarum** and FOS was significantly higher \((P<.05)\) in comparison with that of control yogurt. This might be caused by the efficient proteolytic potential of probiotic lactobacilli, which is improved in the presence of FOS and leads to the liberation of more peptides and amino acids, and ultimately enhances the antioxidant activity of synbiotic yogurt. El-Din et al. (2014) investigated the effect of pomegranate yogurt against hepatic injury (CC14 treatment) in rats and results revealed that pomegranate yogurt improved the level of biomarker scores in serum and liver of CC14-treated rats, and reduced the histopathological changes in liver. Farvin et al. (2010a) studied the antioxidant property of different watersoluble protein fractions of yogurt. The results indicated that there is higher radical scavenging activity in 3–10 kDa and <3 kDa fractions than in other fractions (>30 kDa and 10–30 kDa) because of the presence of low molecular weight peptides, which act as electron donors and could react with free radicals to convert them to more stable products. It was also found that the lower molecular fractions showed protection against oxidation of fish oil-enriched milk emulsion to the same extent as caseinophosphopeptides. In addition, the higher oxidative stability might be caused by antioxidant peptides released during the fermentation of milk by LAB and/or by the lower oxygen content of yogurt, which subsequently reduces the oxidative stress of fish oil incorporated in the milk. The peptides and amino acids responsible for the antioxidant property have been identified by Farvin et al. (2010b). Among the 33 identified peptides, almost all the peptides contained at least one proline residue and hydrophobic amino acid residues Val or Leu at the N-terminus and Pro, His, or Tyr in the amino acid sequence, which is characteristic of antioxidant peptides. In addition, the yogurt contained a considerable amount of free amino acids such as His, Tyr, Thr, and Lys, which have been reported to have antioxidant properties. Yogurt has also been shown to be a good matrix for enrichment with omega-3 fatty acids (Nielsen et al., 2007). A comparison of the oxidative stability of omega-3 fatty acids enriched in milk and yogurt has been studied by Let et al. (2007). The results indicated that yogurt enriched with omega-3 fatty acids had a very good oxidative stability as there is no difference in gross composition between yogurt and milk. It might also be because the compounds liberated during fermentation, namely, peptides and free amino acids, which possess antioxidative properties, play a significant role.

### 24.3.9 Antidiabetic Peptides

Diabetes is a serious, chronic disease that occurs either when the pancreas does not produce enough insulin (a hormone that regulates blood sugar or glucose) or when the body cannot effectively use the insulin it produces. It is an important public health problem, and one of four priority noncommunicable diseases targeted for action by world leaders. Both the number of cases and the prevalence of diabetes have been steadily increasing over the past few decades. Globally, an estimated 422 million adults were living with diabetes in 2014, compared to 108 million in 1980. The global prevalence (age standardized) of diabetes has nearly doubled since 1980, rising from 4.7% to 8.5% in the adult population. In 2012 alone diabetes caused 1.5 million deaths. Its complications can lead to heart attack, stroke, blindness, kidney failure, and lower limb amputation.

Consumption of milk protein-derived bioactive peptides has been linked with serum glucose regulatory properties in humans. Different mechanisms may include an insulinotropic activity, incretin secretagogue action, as well as activity on different metabolic enzymes involved in the regulation of serum glucose such as dipeptidyl peptidase IV, α-amylase, and α-glucosidase (Lacroix and Li-Chan, 2014). Shori and Baba (2013) studied the α-glucosidase and α-amylase activity in plain and *Azadirachta indica* (Neem) yogurt. The results showed that fresh *A. indica* yogurt (44.4% ± 3.0%) had higher...
inhibitory effects on α-amylase than plain yogurt (29.8%±5.3%) on day 0. Refrigerated storage increased plain yogurt α-amylase inhibition during the first 14 days to 47% but this reduced to 23%–28% between the third and fourth week of storage. In the initial storage days, the relatively less specific peptides released during fermentation were further cleaved to smaller and possibly more bioactive peptides during the first 14 days of refrigeration. However, extensive proteolysis of these proteins during extended storage may yield much smaller and less bioactive peptides. The addition of A. indica into yogurt may thus change the manner in which the microbial enzymes affected proteolysis. A. indica yogurt has higher mean α-glucosidase inhibition activity than plain yogurt, but both yogurts showed similar changes in α-glucosidase inhibition activity during refrigerated storage with the highest inhibition (15%) recorded on day 14. Yogurt is a good source of free amino acids, which can directly act at the β-cell level to release insulin through various mechanisms including membrane depolarization and mitochondrial signaling, affecting insulin secretion (Newsholme et al., 2010).

24.3.10 MINERAL-BINDING PEPTIDES

Because of its prevalence worldwide, osteoporosis is considered a serious public health concern. Currently, it is estimated that over 200 million people worldwide suffer from this disease. On the other hand, anemia is a widespread nutritional deficiency particularly common in children and women. These two diseases are more prevalent only because of the deficiency and reduced bioavailability of Ca and Fe. Among the biologically active peptides, CPPs refer to casein-derived phosphorylated peptides and may function as carriers for different minerals, playing an important role in their bioavailability. These CPPs have phosphorus bound via monoester linkages to seryl residues in their structure and contain highly polar acidic sequences of three phosphoserines followed by two glutamic acid residues (SpSpSpEE), which are the binding sites for minerals (Clare and Swaisgood, 2000). CPPs have the ability to both increase food stability and promote health, which makes them potential multifunctional food ingredients. These peptides are capable of maintaining minerals (Ca$^{2+}$, Fe$^{2+}$, Zn$^{2+}$) in a soluble form in the intestine, preventing their precipitation and facilitating their absorption. Affinity is highest for calcium (Ca$^{2+}$) and lowest for iron (Fe$^{3+}$). Because of their anionic properties, CPPs are thought to be resistant to gastrointestinal digestion (Kitts, 2005).

Milk fermentation results in a complete solubilization of calcium, magnesium, and phosphorus, and a partial solubilization of trace minerals that ultimately leads to enhanced mineral bioavailability (Terre, 1985). Calcium and phosphorus were shown to be more bioavailable in yogurt than in milk. Long-term yogurt consumption was shown to be associated with a significant increase in serum-ionized calcium (Balasubramanya et al., 1984). Many animal and human studies have reported the presence of CPPs in vivo following ingestion of milk, fermented dairy products, casein, and crude CPP preparations (Meisel and FitzGerald, 2003). The stomach and intestinal contents of adult humans fed milk or yogurt have been found to contain CPPs (Chabance et al., 1998). Kunda et al. (2012) has identified one peptide sequence ($\alpha_{S2}$CN (43–48)) with mineral-binding property from yogurt.

24.3.11 OPIOID PEPTIDES

Opioid peptides are those peptides having pharmacological similarities to opium. Opioid peptide sequences typically contain Tyr-Gly-Gly-Phe at their N-terminal side, and in the case of a typical opioid peptide, a Tyr residue is found at the N-terminus (Teschemacher et al., 1997). These peptides have
been reported to behave like morphine in the brain. These have been found within milk proteins, both in casein ($\alpha_s1$-, $\alpha_s2$-, $\beta$-, and $\kappa$-casomorphins) and whey protein (lactorphins). However, the major opioid peptides are fragments of $\beta$-casein, called $\beta$-casomorphins (Clare and Swaisgood, 2000). On the other hand, all $\kappa$-casein fragments, known as casoxins, behave as opioid antagonists (Séverin and Wenshui, 2005). Opioid peptides have also been found encrypted within the primary sequence of whey proteins such as lactoferrin, $\beta$-lactoglobulin, and bovine serum albumin (Belem et al., 1999). $\beta$-Casomorphins are resistant to the action of gastrointestinal enzymes (Read et al., 1990) and have been associated with the following activities: antihypertensive, immuno-modulatory, antidepressant, antisecretory, and antidiarrheal activities (Pihlanto, 2001). Opioid peptides are thought to be biologically very potent; potentially, micromolar amounts may be sufficient to exert physiological effects (Meisel and Fitzgerald, 2000).

Nguyen et al. (2014) identified $\beta$-casomorphin-5 and $\beta$-casomorphin-7 in yogurt. Systemic administration of a low dose (1 mg/kg, i.p.) of bovine $\beta$-casomorphin-5 was shown to improve the disturbance of learning and memory in mice (Sakaguchi et al., 2006). In addition, $\beta$-casomorphin-7 was shown to significantly contribute to mucin production from both rat and human intestinal mucin-producing cells using real time-polymerase chain reaction and enzyme-linked immunosorbent assay studies (Zoghbi et al., 2006). Since intestinal mucins play a protective role in the gut, consumption of products containing $\beta$-casomorphin-7 could help to improve intestinal health by preventing the adherence of pathogens to the intestinal surface and thus eliminate the onset of intestinal infections. $\beta$-CN (94–123), a peptide detected in yogurts, regulated the secretion and expression of mucins in HT29-MTX cells. Oral administration of this peptide at the rate of 0.1–100 $\mu$mol/L to rat pups enhanced the number of goblet cells and Paneth cells along the small intestine (Plaisancie et al., 2013).

### 24.3.12 IMMUNO-MODULATORY PEPTIDES

Milk protein hydrolysates and peptides derived from caseins and major whey proteins can enhance immune cell functions, measured as lymphocyte proliferation, antibody synthesis, and cytokine regulation (Gill et al., 2000). Peptides released during milk fermentation with LAB show special interest, because these peptides have been found to modulate the proliferation of human lymphocytes to down-regulate the production of certain cytokines and to stimulate the phagocytic activities of macrophages (Matar et al., 2003). Also it has been suggested that immuno-modulatory milk peptides may alleviate allergic reactions in atopic humans and enhance mucosal immunity in the gastrointestinal tract (Korhonen and Pihlanto, 2003). De Simone et al. (1986) reported that filtered yogurt, which is free of microorganisms, increased interferon-gamma production and natural killer activity of human peripheral blood lymphocytes.

### 24.4 CONCLUSION

The relationship between diet and health is now well known to be one of the key factors in preventing diseases and promoting wellbeing. Indeed, it is on this basis that there has been major growth in the market for functional foods. In recent years, the consumption of yogurt has increased rapidly because this dairy product fulfills many of the current dietary needs. Yogurt has proved to be an excellent vehicle for the production of such functional food, especially when it contains probiotic bacteria. It is
also possible that the beneficial health effects of yogurt can be increased based on the peptides that are
produced during fermentation and storage. But fermentation and storage conditions should be main-
tained in such a way that the yogurt retains the activity of required functional peptides. Such types of
yogurts can also be called a traditional nutraceutical.

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25.1 INTRODUCTION

Foodomics has been defined as a new discipline that studies the food and nutrition domains through the application of advanced omics technologies for improving consumer’s wellbeing, health, and confidence (Herrero et al., 2012). This approach takes advantage of developments in genomics, transcriptomics, proteomics, and metabolomics. At the end of the omics cascade, metabolomics is the platform providing a comprehensive qualitative and quantitative overview of the overall metabolites present in a food system (Laghi et al., 2014). Metabolomics has been widely applied to investigate the biochemical changes related to microbial activity during fermentation. This information can be used to predict the safety, sensory, and nutritional quality of fermented food products (Mozzi et al., 2013). From a molecular perspective, yogurt is considered as a complex food system consisting of hundreds of biomolecules including proteins, lipids, carbohydrates, and many other small metabolites, such as amino acids, organic acids, nucleic acids, fatty acids, minerals, and aroma volatiles responsible for its distinctive flavor characteristics (Tamime and Robinson, 2007). Regarding the range of chemical classes, measurement of all metabolites in yogurt using a single analytical platform is challenging. The application of nuclear magnetic resonance (NMR)-based and hyphenated-mass spectrometry (MS)-based technologies have been shown to be very effective for determining a wide range of metabolites in yogurt as well as other fermented dairy products (Maher and Rochfort, 2014; Mozzi et al., 2013). The key success strategies are the collection of high-throughput data and interpretation of the multidimensional datasets. The outcomes have provided new insights regarding the variations in metabolite profiles of fermented products related to specific types of starter cultures, fermentation processes, and storage conditions (Piras et al., 2013). This chapter highlights the significance of metabolomic studies on yogurt and the possibilities for the application of this technology in assessing and predicting the quality of yogurt.

25.2 FUNDAMENTALS OF METABOLOMICS

The suffix “omics” derives from the Latin “omne,” which means everything, entirety, or totality (Mozzi et al., 2013). This term is actually referring to genomics, transcriptomics, proteomics, and metabolomics.
These methodologies are used to study groups of biomolecules in a global perspective, i.e., genes, transcripts, proteins, and metabolites, respectively, and are even used to define the relationships among them (Mozzi et al., 2013). Metabolomics, also known as **metabolome analysis** or **metabolite profiling**, focuses on the comprehensive characterization of small molecular weight molecules, i.e., metabolites (generally <1500 Da), present in a biological system (Wishart, 2008). Metabolites include a range of intracellular as well as secreted compounds such as oligopeptides, amino acids, nucleic acids, carbohydrates, organic acids, vitamins, polyphenols, alkaloids, and other chemical compounds synthesized by a specific cell or organism. These compounds are involved in the metabolism, and their levels can be regarded as the ultimate response of biological systems to genetic or environmental conditions (Ren et al., 2015). The collective set of metabolites found within a biological system is commonly referred as **metabolome** (Wishart, 2008). Rather than studying individual compounds, metabolomics collects quantitative and semiquantitative data over a large range of metabolites (metabolome) to obtain an overall understanding of the metabolism associated with a specific condition (Ren et al., 2015). In the last decade, metabolomics has been developed from a promising concept to a reproducible and efficient approach that can directly reflect biological events of interest (Yi et al., 2016).

In a metabolomic study, the aim is to integrate information collected through a series of technologies in metabolite separation, detection, identification, and quantification (Mozzi et al., 2013). Among the tasks mentioned, separation and detection are considered the key steps in metabolomic studies (Cevallos-Cevallos et al., 2009). The advanced technologies in analytical chemistry provide a high-throughput characterization of hundreds of metabolites in a single measurement. Although there is no analytical instrument that can determine all of the metabolites in a biological system, NMR spectroscopy and hyphenated-MS are the most predominant technologies being used for generating metabolite profiles (Wishart, 2008). NMR spectroscopy can be used to identify and quantify metabolites from complex mixtures (Smolinska et al., 2012). It relies on the nuclei of specific atoms that possess a magnetic spin and when placed inside a magnetic field can adopt different energy levels that can be observed using radiofrequency waves (Ren et al., 2015). NMR spectroscopy is a nondestructive method requiring limited sample preparation (Ren et al., 2015). Another major advantage of NMR is that the signal frequencies observed in an NMR spectrum are directly proportional to the concentration of the nuclei in the sample (Smolinska et al., 2012), allowing the straightforward acquisition of quantitative data. Compared to MS-based technologies, however, NMR exhibits a lower sensitivity by which only medium to high abundance metabolites will be detected (Smolinska et al., 2012). Application of MS coupled with separation methods such as gas chromatography (GC), liquid chromatography (LC), high-performance liquid chromatography, and ultrahigh-pressure liquid chromatography in metabolomic studies is well acknowledged in the field of biological and biochemical research (Jiménez-Pranteda et al., 2014). The advantage of MS-based analytical approaches is that they allow for separation and detection of a wide range of metabolites at low concentrations and at the same time structural information of the detected molecule is obtained (Ren et al., 2015). An overall comparison of advantages and disadvantages of NMR-based and MS-based technologies applied in food and nutritional metabolomics is reviewed by Wishart (2008) and Johanningsmeier et al. (2016). In addition to the two major analytical platforms mentioned earlier, capillary electrophoresis coupled with MS and detection techniques such as Fourier transform infrared spectroscopy and near-infrared spectroscopy can also be employed (Jiménez-Pranteda et al., 2014). Review articles on high-throughput technologies and their suitability in metabolomic analysis have been extensively published (Cubero-Leon et al., 2014; Ibañez et al., 2013; Laghi et al., 2014;
Mozzi et al., 2013; Ren et al., 2015). Thanks to these advanced analytical platforms, the creation of databases containing spectral data and the corresponding signal assignments has driven the progress in metabolomic research (Capozzi and Trimigno, 2015).

25.3 STATISTICAL ANALYSIS AND INTERPRETATION OF METABOLOMIC DATA

In general, metabolomic approaches employ either targeted or nontargeted analyses (Yi et al., 2016). Targeted analysis focuses on a group of metabolites that requires specific identification and quantification processes. This analysis is important for assessing the behavior of a particular collection of metabolites under certain conditions. However, this approach has a limitation in terms of the extensiveness of analysis. It typically requires a higher level of purification and selective extraction of metabolites prior to analysis (Cevallos-Cevallos et al., 2009). The data obtained are relatively simple and often suitable for univariate statistics or variable selection and modeling (Yi et al., 2016). On the other hand, nontargeted analysis focuses on the detection of as many groups of metabolites as possible. This approach allows the discovery of the overall metabolite profiles or fingerprints of the samples without necessarily identifying or quantifying any specific compounds (Cevallos-Cevallos et al., 2009). The aim is to compare metabolite profiles of biological systems in response to a specific circumstance, e.g., genetic variation or specific environmental conditions (Cuadros-Rodríguez et al., 2016).

It should be mentioned that omics analyses in general are based on the analysis of a large quantity of information by applying bioinformatics tools for data interpretation (Mozzi et al., 2013). For that reason, the key success strategies for metabolomics are (1) collection of high-throughput (metabolome) data and (2) interpretation of multidimensional datasets that require combined knowledge of advanced analytical chemistry and chemometrics (Blow, 2008). Chemometrics is a multidisciplinary approach, specifically applied to chemical datasets, that combines multivariate statistical analysis, mathematical modeling, and information technology (Cruz et al., 2013). The application of multivariate statistical analysis enables the identification of possible correlations, covariations, and patterns among samples (Mannina et al., 2012), thereby allowing classification of samples into one or more groups of biological significance (Johanningsmeier et al., 2016). In general, there are two approaches to process metabolomic data, i.e., (1) unsupervised and (2) supervised methods (Ren et al., 2015). Unsupervised methods are usually applied to explore the overall structure of a dataset, aimed at finding trends and clustering samples within the dataset. These methods provide an unbiased overview of the data. Several unsupervised methods are available, in which principal component analysis, hierarchical cluster analysis, and self-organization mapping are the most commonly used algorithms in metabolomic studies (Yi et al., 2016). Supervised methods incorporate a priori acknowledged data structures into the models to discover biomarkers, patterns, and rules to classify new data. Supervised methods can be classified as (1) linear methods, such as partial least squares discriminant analysis and orthogonal projections to latent structures discriminant analysis and (2) nonlinear methods, such as random forest and support vector machine (Yi et al., 2016). One challenge for using multivariate statistics for metabolomic data is the generally small sample size, requiring strategies to prevent overfitting, especially when used for supervised classification techniques. Further overviews of multivariate statistical analysis of metabolomic data can be found elsewhere (Ren et al., 2015; Skov et al., 2014; Wu and Wang, 2015; Yi et al., 2016).
25.4 METABOLOMICS AS PART OF THE STRATEGIES IN FOODOMICS

Metabolomics provides, along with genomics, transcriptomics, and proteomics, the holistic definition of food according to the new foodomics concept, which has been defined as a new discipline that studies the food and nutrition domains through the application of advanced omics technologies to improve consumers’ wellbeing, health, and confidence (Cifuentes, 2013). Progressively, metabolomics has been applied to food and nutrition research (Castro-Puyana and Herrero, 2013). The complete collection of small molecules present in food, commonly referred to as food metabolome, mainly comprises metabolites from animals, plants, and microorganisms, which may be further altered by primary production, processing, storage, as well as unintentional contamination (Johanningsmeier et al., 2016). Variations in abundance of these compounds may directly affect the quality and nutritional characteristics of food. Metabolomics provides opportunities to discover and establish new biochemical pathways, metabolite databases, molecular profiles, potential biomarkers, and bioactive compounds, which can be directly correlated with the quality, nutritional value, safety, fermentation, processing, storage, traceability, and authenticity of raw materials and final food products (Castro-Puyana and Herrero, 2013; Cevallos-Cevallos et al., 2009; García-Cañas et al., 2012; Herrero et al., 2012; Mozzi et al., 2013; Wishart, 2008). This turns into a promising approach to rapidly evaluate the quality of food products.

25.5 METABOLOMICS APPLIED IN MILK AND FERMENTED DAIRY RESEARCH

Milk is a complex biological fluid comprising several components present in various physicochemical states, i.e., emulsion, suspension, and solution (Scano et al., 2014). The principal components of bovine milk are on average of 87.1% water, 3.3% protein, 4.0% fat, 4.6% lactose, 0.7% mineral substances, and 0.3% organic acids and miscellaneous (Walstra et al., 2006). These contents, especially those of fat and protein, are considerably influenced by the breed of the cow, stage of lactation, age, physiological status, type of feed, farming methods, time of year, and milk processing (Walstra et al., 2006). These factors may have significant influences on the physicochemical aspects, technological properties, and nutrient density of milk (Scano et al., 2014). For that reason, the price of raw milk is determined based on quantitative assessment of these major components in combination with hygienic quality. Recently, the application of metabolomics has been well acknowledged in dairy science and technology research. Studies focusing on variation in milk metabolites, especially for the impact on animal species, feed, farm management, and environmental conditions, have been extensively published. Up to now, over 200 metabolites including amino acids, lipids, carbohydrates, nucleotides, energy metabolites, vitamins, cofactors, and short peptides have been identified in liquid milk using NMR-based and MS-based technologies (Boudonck et al., 2009; Klein et al., 2010; Sundekilde et al., 2013). This information can be used to establish a correlation between the metabolite profile of milk and factors influencing its variability (Lamanna et al., 2011). For example, influences of genetic variability on milk metabolites have been investigated (Lu et al., 2015; Wittenburg et al., 2013). Lu et al. (2015) reported a study of milk samples from dairy cows with different alleles of the DGAT1 gene encoding an enzyme that catalyzes the synthesis of triglycerides from diglycerides and acyl-coenzyme A. The so-called DGAT1-KK genotype contained more stomatin, sphingomyelin, choline, and carnitine, and less citrate, creatine or phosphocreatine, glycerol-phosphocholine, mannose-like sugar, acetyl sugar phosphate, uridine diphosphate...
(UDP)-related sugar, and orotic acid compared with milk from cows with the DGAT1-AA genotype. The authors discussed that this difference may be related to stomatin–sphingomyelin lipid rafts as well as structural differences in epithelial cells of the mammary gland. Also associations between milk metabolites and the physiological and health status of dairy cows, i.e., dry period length, energy balance, lactation stage, heat stress, pathogenesis including mastitis, and ketosis, have been extensively documented (Antunes-Fernandes et al., 2016; Lu et al., 2013; Melzer et al., 2013; Silanikove et al., 2014; Sun et al., 2015; Tian et al., 2016). For example, biomarkers such as acetone and β-hydroxybutyrate were reported to be correlated with the metabolic status of cows during early lactation stage (Klein et al., 2010). Correlation between milk metabolome and methane emission in dairy cows was reported by Antunes-Fernandes et al. (2016). The authors found that the volatile metabolites 1-heptanol-decanol, 3-nonanone, ethanol, and tetrahydrofuran determined by GC/MS were positively correlated with methane intensity. Furthermore, the nonvolatile metabolites acetoacetate, creatinine, ethanol, formate, methylmalonate, and N-acetyl sugar A were positively related to methane intensity, whereas UDP-hexose B and citrate were negatively related to methane intensity. These results suggest metabolic alterations in the mammary gland of dairy cows in relation to methane emission. The analysis of milk metabolites using the LC/MS technique demonstrated that lactate, pyruvate, creatine, acetone, β-hydroxybutyrate, trimethylamine, oleic acid, linoleic acid, lysophosphatidylcholine 16:0, and phati-dylcholine 42:2 showed strong correlations with heat stress status of dairy cows (Tian et al., 2016). The possibility of detecting clinical mastitis caused by Staphylococcus aureus, coagulase-negative staphylococci, Streptococcus uberis, Streptococcus dysgalactiae, and Escherichia coli according to their volatile metabolite formation in raw milk was revealed by Hettinga et al. (2008). Regarding agricultural practices, studies assessing differences in the metabolite profile of organic and conventional milk have been published (Boudonck et al., 2009; Erich et al., 2015). Boudonck et al. (2009) found that tyrosine, isoleucine, mannose, glycerate, ribose, carnitine, butyrylcarnitine, and hippurate were present at higher concentrations in organic milk compared to conventional milk. The authors discussed that a particular increase in hippurate and its derivative, i.e., 2-hydroxyhippurate, were positively linked to dietary fiber intake. This could be a direct reflection of different diets between organically and conventionally raised dairy cows (Boudonck et al., 2009). An investigation of indicative metabolites (biomarkers) in raw milk from ruminants (cow, buffalo, yak, and goat) and nonruminants (camel and horse) using NMR and LC/MS techniques was performed by Yang et al. (2016). The authors stated that choline and succinic acid could be used as biomarkers to distinguish Holstein cow’s milk from that of the other studied animals. Also these authors found that two pathways including glycerophospholipid metabolism and valine, leucine, and isoleucine biosynthesis were a common attribute in the ruminant animals. A metabolomic investigation of goat’s milk compared with bovine milk and the effect of different thermal treatments using the GC/MS technique was performed by Scano et al. (2014). The authors reported that valine and glycine were specific to goat’s milk, talose and malic acid to cow’s milk, and hydroxyglutaric acid to pasteurized milk samples. Glucose and fructose were shared by cow’s milk and UHT-treated samples, whereas ribose was shared by pasteurized and goat’s milk (Scano et al., 2014).

An application of proton time domain NMR as a rapid nondestructive method for detecting adulterated milk with whey, urea, hydrogen peroxide, artificial urine, and synthetic milk prepared by emulsifying vegetable oils was reported by Santos et al. (2016). The outcomes from the aforementioned studies provide insights regarding the variation in metabolites present in liquid milk influenced by particular agricultural practices, animal physiology and health status, thermal process, as well as chemical adulteration.
Regarding fermented dairy products, metabolomics has been successfully applied to evaluate the metabolite profile of fermented milk and cheeses in association with coagulation properties and microbial activity during fermentation, ripening, and storage (Mozzi et al., 2013; Zheng et al., 2015). Pisano et al. (2016) evaluated the metabolite profile of mozzarella cheese produced from cow’s and buffalo milk using the GC/MS technique. The authors found that buffalo mozzarella was significantly higher in the concentration of threonine, serine, and valine, and lower in the concentration of orotic acid and urea. The production of volatile metabolites by various strains of Lactobacillus spp. and Leuconostoc spp. in cheese conditions was determined by Pogacic et al. (2016) using the headspace GC/MS technique. The authors discovered very large differences in the concentration of volatile metabolites between the highest producing strains and the control medium, particularly for diacetyl, 2-butanol, ethyl acetate, 3-methylbutanol, 3-methylbutanoic acid, and 2-methylbutanoic acid. The study of Harzia et al. (2012) demonstrated that carnitine and oligosaccharides was correlated with the acid coagulation ability of milk. Sundekilde et al. (2014) reported that the level of lactate, acetate, glutamate, creatinine, choline, carnitine, galactose-1-phosphate, and glycerophosphocholine was significantly different between noncoagulating and rennet-induced coagulating milk. A metabolite analysis of milk fermented by γ-aminobutyric acid (GABA)-producing Lactococcus lactis was performed by Hagi et al. (2016) using capillary electrophoresis time-of-flight mass spectrometry (CE-TOF-MS). Their results indicated that the GABA-producing L. lactis strain provided fermented milk with a higher concentration of bioactive peptides and free amino acids. This suggests an additional functionality of the product. GC-TOF-MS was used to perform volatile metabolite profiling in milk by various combinations of lactic acid bacteria including starters used for making yogurt (De Bok et al., 2011). The authors were able to discriminate different species and mixtures of lactic acid bacteria based on volatile metabolites produced in milk. Furthermore, differences in flavor production levels may be linked to strain-specific genetic markers when genomic data are available. Benozzi et al. (2015) monitored volatile metabolites formed during lactic acid fermentation by the activity of different yogurt starters using proton transfer reaction time-of-flight mass spectrometry. This analytical technique measures the time-dependent evolution of several mass peaks of respective metabolites, both with increasing and decreasing trends. The authors highlighted specific depletion kinetics for sulfur compounds, i.e., hydrogen sulfide, methanethiol, S-methyl thioacetate/S-ethyl thioformate, and pentanethiol, in yogurt during fermentation, proposing a new technological role for starter cultures in yogurt.

From a molecular point of view, yogurt is considered as a complex food system consisting of hundreds of biomolecules including proteins, lipids, carbohydrates, and many other small metabolites, such as amino acids, organic acids, nucleic acids, fatty acids, minerals, and various aroma volatile compounds responsible for its distinctive flavor characteristics (Tamime and Robinson, 2007). Regarding the range of chemical classes, measurement of all metabolites present in yogurt using a single analytical technique appears to be unattainable. A complementary metabolomic approach using headspace solid-phase microextraction (SPME)-GC/MS and 1H-NMR has been applied for determining a global metabolite profile, i.e., accounting for volatile and nonvolatile metabolites present in yogurt (Settachaimongkon, 2014). Finally, metabolite profiles of different yogurt samples were statistically compared by means of multivariate analysis (Fig. 25.1). The outcomes provided new insights regarding the variations in metabolite profile of yogurt related to the activity of starter cultures and the presence of probiotic strains during fermentation and refrigerated storage.
METABOLICS TO INVESTIGATE THE PROTOCOOPERATION

According to the Codex standard for fermented milk (CODEX, 2003), yogurt is specifically characterized by the presence of symbiotic cultures of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*. During fermentation, these bacteria perform three major biochemical conversions of milk components: (1) conversion of lactose into lactic acid (fermentation), (2) hydrolysis of caseins into peptides and free amino acids (proteolysis), and (3) breakdown of milk fat into free fatty acids (lipolysis) (Smit et al., 2005). These reactions lead to the production of various metabolites resulting in a decrease of the pH, formation of a semisolid texture, and a distinctive yogurt flavor (Irigoyen et al., 2012). Complete genome sequences and functional-genomic analyses of many *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* strains have been published (Bai et al., 2016; Bolotin et al., 2004; Delorme et al., 2011; Hao et al., 2011; Kaladhar and Ramakrishna, 2013; Kang et al., 2012; Sun et al., 2011a,b; Van de Guchte et al., 2006).

Although *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* are able to grow independently in milk, these bacteria perform a symbiotic interaction known as *protocooperation* in mixed culture (Courtin and Rul, 2004; Tamime and Robinson, 2007). Protocooperation is based on the exchange of several metabolites that provide growth-stimulating effects to each other (Smid and Lacroix, 2013). Traditional yogurt starters consist of a weakly or nonproteolytic *S. thermophilus* strain combined with a proteolytic...
L. delbrueckii ssp. bulgaricus strain (Walstra et al., 2006). During the early stage of fermentation, the initial pH of milk (c. 6.7) is more favorable to the growth of S. thermophilus. These bacteria develop by using free amino acids and peptides available in milk. However, the contents of these free nitrogen sources are not sufficient to promote their full growth (Letort and Juillard, 2001). Next, S. thermophilus utilizes amino acids and peptides delivered in the milk because of the proteolytic activity of L. delbrueckii ssp. bulgaricus. On the other hand, S. thermophilus produces lactic acid that consequently lowers the pH, hence retards its growth, and creates a favorable growth condition for L. delbrueckii ssp. bulgaricus. Furthermore, pyruvic acid, formic acid, folate, ornithine, several long-chain fatty acids, and CO$_2$ produced by S. thermophilus stimulate the growth of L. delbrueckii ssp. bulgaricus (Angelov et al., 2009; Sieuwerts et al., 2008; Tari et al., 2009; Zourari et al., 1992). Protocooperation has an important role in the growth of the two species, rate of milk acidification, and development of distinctive flavor and texture characteristics of the fermented product (Chandan, 2006; Zourari et al., 1992). Although interaction between the two species is often positive (protocooperation), the absence of interactions or even negative effects (antagonism) (Smid and Lacroix, 2013) can take place depending on the combination of bacterial strains, type and heating process of base milk, and fermentation conditions (Courtin and Rul, 2004). Therefore, selection of suitable strain combinations in yogurt starters is important for achieving the best technological performance regarding the quality of products (Settachaimongkon et al., 2014a).

Postgenomic studies, i.e., transcriptomics and proteomics, in mixed cultures of S. thermophilus and L. delbrueckii ssp. bulgaricus have revealed new insights in physiology and molecular basis of the interaction (Hao et al., 2011; Herve-Jimenez et al., 2009; Liu et al., 2009; Oliveira et al., 2012; Pastink et al., 2009; Sieuwerts et al., 2008, 2010). Sieuwerts et al. (2008) demonstrated how genomic approaches may contribute to the elucidation of the interaction networks between S. thermophilus and L. delbrueckii ssp. bulgaricus, including interactions with the fermented food environment. Herve-Jimenez et al. (2009), Sieuwerts et al. (2010), and Thevenard et al. (2011) performed transcriptomic and proteomic analyses of S. thermophilus in response to the presence of L. delbrueckii ssp. bulgaricus during mutual growth in milk. The results of Sieuwerts et al. (2010) indicated that interactions between these bacteria were primarily related to purine, amino acid, and long-chain fatty acid metabolism. This supports a model in which formic acid, folate acid, and fatty acids are provided by S. thermophilus. Proteolysis by L. delbrueckii ssp. bulgaricus supplies both strains with amino acids but is insufficient to meet the biosynthetic demands for sulfur and branched-chain amino acids. The study of Sieuwerts et al. (2010) clarified this point from the upregulation of genes associated with these amino acids, i.e., prtB-LBUL-1105 gene encoding the extracellular protease activity, in mixed culture. Several industrial features of S. thermophilus such as the unique pathway for acetaldehyde production, amino acid metabolism, and amino acid dependency were identified by using genome-scale metabolic modeling (Pastink et al., 2009). Proton transfer reaction-mass spectrometry has been applied to monitor lactic fermentation and flavor volatile compounds formed by yogurt starter cultures (Benozi et al., 2015). De Bok et al. (2011) used ultrafast gas chromatography coupled to time-of-flight mass spectrometry to perform untargeted profiling of volatile compounds formed by different mixed cultures of lactic acid bacteria including S. thermophilus and L. delbrueckii ssp. bulgaricus. The random forest algorithm was able to sort out mass peaks originated from key flavor compounds that discriminated combinations of species or strains used in fermentation. Settachaimongkon et al. (2014a) investigated the protocooperation between proteolytic and nonproteolytic strains of S. thermophilus with L. delbrueckii ssp. bulgaricus in yogurt fermentation. A complementary metabolomic approach using headspace SPME-GC/MS and
H-NMR was applied for the global characterization of volatile and nonvolatile polar metabolite profiles of yogurt associated with proteolytic activity of the individual strains in the starter cultures. The headspace SPME-GC/MS technique provides a good method for determination of aroma volatile compounds in yogurt. A total of 35 compounds consisting of alcohols, carbonyl compounds, organic acids, sulfur compounds, and heterocyclic compounds were identified in yogurt fermented by different types of starter cultures. Their results demonstrated the associative volatile production between the nonproteolytic strains of S. thermophilus with L. delbrueckii ssp. bulgaricus. Protocooperation between these two strains resulted in the highest numbers of volatiles identified with significant abundance of key aroma compounds, i.e., acetaldehyde (fresh, green, pungent), diacetyl (buttery, creamy), acetoin (buttery), 2-butanone (sweet, fruity), 2,3-pentanediione (buttery, vanilla-like), and acetic acid (vinegar, pungent) (Cheng, 2010), desirable for a good organoleptic quality of yogurt (Fig. 25.2).

Also in the study of Settachaimongkon et al. (2014a), a total of 43 nonvolatile polar metabolites including amino acids, carbohydrates, organic acids, lipid derivatives, carbonyl compounds, a sulfur compound, and a nucleoside were presumptively identified using 1D-NOESY-1H-NMR spectroscopy (Fig. 25.3). The influence of bacterial proteolytic activity has been characterized by an overall increase in concentration of free amino acids in the growth medium. The 1H-NMR measurement revealed that protocooperation between the nonproteolytic S. thermophilus and L. delbrueckii ssp. bulgaricus provided not only a growth stimulatory effect on the two species but also activated the proteolytic activity of L. delbrueckii ssp. bulgaricus resulting in a significantly higher concentration of free amino acids. The authors concluded that 1H-NMR has several advantages for the determination of nonvolatile polar metabolites in yogurt such as minimal pretreatment required and simultaneous measurement of all polar metabolites. Furthermore, the combination of metabolomics-based data with multivariate statistical analysis allows the discrimination of yogurt samples fermented by different types of starter cultures according to their volatile and nonvolatile polar metabolite profiles.

25.7 METABOLICOMICS TO ANALYZE THE IMPACT OF INCORPORATING PROBIOTICS IN YOGURT

Functional foods are defined as foods that potentially provide health benefits in addition to the fundamental nutrients they contain (Shiby and Mishra, 2013). One way in which foods can be modified to become functional is by the addition of health-associating microorganisms referred as probiotics (Shah, 2007). The term probiotics originates from Greek meaning “for life” (Fuller, 1992). According to the Food and Agriculture Organization of the United Nations/World Health Organization Guidelines for the evaluation of probiotics in food, probiotics are defined as live microorganisms that, when administered in adequate amounts, confer a health benefit on the host (FAO/WHO, 2002; Hill et al., 2014). Several criteria need to be considered in the selection of probiotic microorganisms including clinical safety, efficacy, functional properties, and technological characteristics (Saarela et al., 2000). Most commercial probiotics incorporated in dairy products are strains belonging to the genera Lactobacillus and Bifidobacterium (Lourens-Hattingh and Viljoen, 2001). Members of these two genera have a long history of safe use in the manufacture of fermented foods and are part of normal microbiota in the human gastrointestinal tract (Shah, 2000). A number of health benefits are claimed in favor of the consumption of probiotic Lactobacillus and Bifidobacterium including modulation of the immune system, prevention and reduction of gastrointestinal disorders, alleviation of lactose intolerance, prevention of
Heat map and hierarchical clustering of volatile metabolite profiles from Nilac milk and set yogurts fermented by different types of starter cultures. Dendrogram represents sample clusters based on Pearson’s correlation coefficient with average linkage. Each square in the heat map expresses normalized volatile content with respect to the color range. The red (gray in print versions) color indicates higher content of the corresponding compound. For better visualization of the referred color, the reader is recommended to visit the online version of this chapter.

allergies, reduction of the risks associated with mutagenicity and carcinogenicity, inhibition of intestinal pathogens, prevention of inflammatory bowel disease, and reduction in serum cholesterol (Granato et al., 2010; Sánchez et al., 2009; Shibly and Mishra, 2013; Vasiljevic and Shah, 2008). However, it should be mentioned that the positive health effects imparted by probiotics are strain specific. Among the probiotic strains incorporated in dairy products, *Lactobacillus rhamnosus* GG (Valio), *Lactobacillus casei* Shirotta (Yakult), and *Bifidobacterium animalis* ssp. *lactis* BB12 (Chr. Hansen) are extensively studied strains with the most clinically documented effects in humans (Shah, 2007).

**FIGURE 25.3**

Representative NOESY 1D-1H-NMR spectra of a set yogurt sample fermented by a mixed culture of *Streptococcus thermophilus* protease (−) and *Lactobacillus delbrueckii* ssp. *bulgaricus* (A) and expansions corresponding to the aliphatic region (B), sugar region (C), and aromatic region (D) with assigned peaks: TSP, internal standard; 1, valerate derivatives; 2, valerate; 3, butyrate; 4, isoleucine; 5, leucine; 6, valine; 7, isobutyrate; 8, lactate; 9, alanine; 10, acetate; 11, N-acetyl amino acids; 12, N-acetyl glucosamine; 13, acetone; 14, acetoacetate; 15, proline; 16, pyruvate; 17, succinate; 18, oxoglutarate; 19, citrate; 20, creatine; 21, creatinine; 22, dimethyl sulfone; 23, acetyl carnitine; 24, choline derivatives; 25, betaine; 26, glucose; 27, lactose; 28, galactose; 29, ascorbate; 30, choline; 31, phosphocholine; 32, glycerophosphocholine; 33, dihydroxyacetone; 34, sugar residues; 35, uridine; 36, orotate; 37, fumarate; 38, amino acid residues; 39, tyrosine; 40, phenylalanine; 41, benzoate; 42, hippurate; 43, formate.

*Reprinted from Settachaimongkon, S. Influence of different proteolytic strains of Streptococcus thermophilus in co-culture with Lactobacillus delbrueckii subsp. bulgaricus on the metabolite profile of set-yoghurt. Simultaneous Growth and Metabolite Production by Yoghurt Starters and Probiotics: A Metabolomics Approach, pp. 35–65, Copyright (2014), with permission from Wageningen University.*
Advanced omics technologies provide a range of high-throughput methods that are extremely useful for unraveling the beneficial effects of probiotics, allowing a rational selection of strains with specific health-promoting activities to be implemented in functional foods (Sánchez et al., 2013). Overviews of advancements in complete genome sequencing and functional genomic analyses in various probiotic strains have been extensively published (Johnson and Klaenhammer, 2014; Papadimitriou et al., 2015b; Sánchez et al., 2013; Siezen and Wilson, 2010). For example, Smid and Hugenholtz (2010) reviewed the application of functional genomics in food fermentation processes. Papadimitriou et al. (2015b) described how omics technologies allow exploration of novel routes for screening and studying probiotics. The work of Jiménez-Pranteda et al. (2014) provided a summary of the omics application for validating models of interactions among gut microbiota, probiotics, and human health. Douillard and de Vos (2014) published an overview of the application of functional genomic approaches and their impact on designing lactic acid bacteria for food and health. Siciliano and Mazzeo (2015) discussed the applications of proteomics to elucidate the molecular mechanisms of probiotics in association with their adhesion to the human gastrointestinal tract as well as immunomodulation. Sánchez et al. (2013) discussed how omics are contributing to understanding the functions of probiotics and their mechanisms of action. Achievements of metabolomics in the comprehensive analysis of foods fermented by lactic acid bacteria, the fermentative capacity of these microorganisms, and the beneficial effects of functional foods and probiotics have been reviewed by Mozzi et al. (2013). Compared to yogurt starters, however, there is still limited information regarding the metabolic activity of probiotics in milk (Plessas et al., 2012). This information is important, since the organic acids and volatile compounds formed by these functional bacteria may directly influence the organoleptic quality of products (Østlie et al., 2005). As mentioned earlier, Hagi et al. (2016) used the CE-TOF-MS technique to find specific components in milk fermented with GABA-producing L. lactis. Pogacic et al. (2016) evaluated the potential of Leuconostoc spp. and Lactobacillus spp. in the production of aroma compounds by metabolic fingerprints of volatiles using the headspace trap GC/MS technique. In the study of Settachaimongkon et al. (2014b, 2016), a complementary metabolomic approach, using headspace SPME-GC/MS and 1H-NMR, was applied to evaluate the impact of selected strains of probiotics on metabolite formation in set yogurt. The works of these authors revealed that incorporation of L. rhamnosus GG, B. animalis ssp. lactis BB12, and Lactobacillus plantarum WCFS1 did not significantly influence the acidity and concentrations of key aroma volatile compounds of set yogurt. Still, all probiotic strains had a significant impact on the overall yogurt metabolite profiles. The presence of probiotics substantially contributed to the formation of a large number of volatile and nonvolatile metabolites detected at low concentration. Variation in the overall metabolite profiles of set yogurts cofermented with different probiotic strains could be statistically determined using multivariate analysis. Moreover, the authors concluded that the combination of metabolomics-derived data with multivariate analysis allowed discrimination of yogurt samples according to the difference in types of starter combinations, together with durations of storage (Fig. 25.4).

25.8 METABOLOMICS TO INVESTIGATE THE ACTIVITY OF PRESTRESS-ADAPTED PROBIOTICS IN YOGURT

The definition of probiotics underlines that these functional bacteria need to be viable, metabolically active, and present in sufficiently high numbers at the time of consumption to ensure their beneficial health effects (FAO/WHO, 2002). It is recommended that a probiotic product should contain at least
10^6 cfu/g of viable probiotic cells throughout its entire shelf-life (Vasiljevic and Shah, 2008). However, numerous studies have demonstrated that many probiotic strains are not able to survive well in fermented milk (Donkor et al., 2007; Gueimonde et al., 2004; Saccaro et al., 2009; Shah, 2000). The survival of probiotics can be adversely affected by certain metabolites including lactic acid, hydrogen peroxide, and bacteriocins produced by yogurt starters (Mohammadi et al., 2012). Besides this, other...
factors accountable for the viability loss of probiotics during yogurt manufacturing and storage, including sensitivity of the strains used, inoculation rate and level, fermentation temperature, level of oxygen permeation through the package, presence of other competitive lactic acid bacteria, and application of food additives, have been extensively reported (Mohammadi et al., 2012; Plessas et al., 2012; Shah, 2007).

Certain approaches have been applied for improving the survival of probiotics during yogurt manufacturing and storage. The most prevalent are (1) the selection of appropriate strains on the basis of their acid and bile tolerances, (2) supplementation of the milk with nutrients, (3) addition of protective compounds, (4) manipulation of starter cultures, (5) selection of appropriate packaging materials, (6) application of oxygen scavengers, (7) performing two-stage fermentation, and (8) application of microencapsulation techniques (Efiovwevwere et al., 1999; Sarkar, 2010; Shah, 2000; Tamime, 2005).

An alternative strategy to improve the survival of probiotics in yogurt is to enhance their ability to cope with harsh environments during manufacturing and storage. Stress adaptation is one of the strategies to improve the survival of probiotics by pretreating (preculturing) them in a sublethal stress condition prior to exposure to a more harsh or lethal environment (Upadrasta et al., 2011). This approach allows probiotic cells to develop adaptive stress responses, i.e., a phenotypic reaction to growth inhibition induced by environmental or physiological imbalances (De Dea Lindner et al., 2007), leading to an increase in their survival compared to those that are directly shifted into the same lethal stress condition (Saarela et al., 2004). Adaptive responses to various types of stress, i.e., heat, cold, acid, bile, osmotic, oxygen, high pressure, and nutrient starvation, in Lactobacillus and Bifidobacterium have been well investigated (De Angelis and Gobbetti, 2004; Ruiz et al., 2011; Tsakalidou and Papadimitriou, 2011; Van de Guchte et al., 2002). These stress conditions are characterized because of the environmental challenges where probiotics are typically encountered, i.e., during human gastrointestinal transit, industrial-scale production, and in the food systems (Ruiz et al., 2011). An overview of the stress factors threatening probiotic viability in foods is given by Capozzi et al. (2015).

Acid and osmotic stress, as consequences of lactic acid production and application of food additives, are the most predominant stress factors during yogurt manufacture and refrigerated storage (Mohammadi et al., 2012). Improved survival of probiotics under acidic conditions is induced by a physiological adaptation known as acid tolerance response (ATR) (Van de Guchte et al., 2002). The ATR associated mechanisms primarily include (1) pH homeostasis by proton-translocating F$_1$F$_0$-ATPase, (2) alteration of cell membrane properties by modification of the fatty acid composition, (3) increase of alkalinity of cytoplasm by the activity of arginine deiminase, urease, and glutamine decarboxylase, and (4) production of several stress proteins (De Angelis and Gobbetti, 2004; Ruiz et al., 2011; Van de Guchte et al., 2002). The response to osmotic stress results in the accumulation of compatible solutes and activation of membrane-associated proteins for maintaining turgor pressure of the cell (Serrazanetti et al., 2009). As a consequence, stress adaptation not only enables the enhancement of the survival of probiotics but also induces substantial changes in their performance in a food system (Serrazanetti et al., 2009).

Advances in postgenomic technologies have extensively provided novel insights into how probiotics counteract environmental stresses (Papadimitriou et al., 2015a; Sánchez et al., 2013). Applications of transcriptomic and proteomic analyses to understand adaptive stress responses in probiotic Lactobacillus and Bifidobacterium have been extensively published (An et al., 2014; De Angelis et al., 2016; Hosseini Nezhad et al., 2015; Hussain et al., 2013). However, there are only a limited number of studies investigating the fate of stress-adapted bacteria when administered in a real food system such
as milk and yogurt (Johnson and Klaenhammer, 2014; Papadimitriou et al., 2015b). The studies of Settachaimongkon et al. (2015, 2016) revealed adaptive stress responses of *L. rhamnosus* GG, *B. animalis* ssp. *lactis* BB12, and *L. plantarum* WCFS1 to sublethal salt and low pH stress conditions exposed during preculturing. Their finding supports the hypothesis that preadaptation can enhance the survival of probiotics in a food system (Settachaimongkon et al., 2015). Besides the direct impact on the viability of probiotics, an adverse effect of stress-adapted *L. plantarum* WCFS1 on the survival of *L. delbrueckii* ssp. *bulgaricus* was observed in the study of Settachaimongkon et al. (2016). This consequently provided a significant reduction of postacidification of the yogurt during refrigerated storage.

It is well documented that environmental stresses induce alterations in the metabolic activity of probiotics leading to substantial changes in their technological and functional performances (Ruiz et al., 2011; Serrazanetti et al., 2009; Tsakalidou and Papadimitriou, 2011). It has also been documented that the ATR in *Lactobacillus* and *Bifidobacterium* is associated with certain metabolic changes, especially the function of enzymes involved in glycolysis and pyruvate metabolism (Ruiz et al., 2011; Sánchez et al., 2012). The results of Settachaimongkon et al. (2015, 2016) revealed that acetic acid, acetoin, 2-butanone, ethanol dimethyl sulfide, 3-methyl-2-butenal, acetic acid, 2-ethyl-hexanol, 1-butanol, 3-methyl-3-butanol, and nonanoic acid were accountable for the separation of yogurt samples cofermented with sublethally precultured probiotics. An increase in the production of these metabolites could be correlated with a higher yield of ATP for supporting the pH homeostasis by F$_1$F$_0$-ATPase (Sánchez et al., 2007). Furthermore, a higher concentration of several enzymes involved in the biosynthesis of branched-chain amino acids as well as sulfur amino acids was reported to be associated with ATR (Sánchez et al., 2007). Also the results of these authors showed an effect of sublethally precultured probiotics on the content of various volatiles derived from the catabolism of these amino acids in yogurt, i.e., 1-methoxy-2-propanol (Val), 2-methyl-1-butanol (Ile/Leu), 3-methyl-2-butenal (Ile/Leu), 3-methyl-butyanoic acid (Leu), 2-methyl-propanoic acid (Val), and sulfur compounds (Cys/Met) (Settachaimongkon et al., 2015). Furthermore, the impact of metabolic activity of stress-adapted probiotics on the volatile and nonvolatile metabolite profile of yogurt was clearly demonstrated by multivariate statistical analysis. Volatile metabolite profiles of the samples cofermented with sublethally precultured *Lactobacillus*, i.e., *L. rhamnosus* GG and *L. plantarum* WCFS1, could be distinguished based on the salt stress levels, whereas samples cofermented with sublethally precultured *B. animalis* ssp. *lactis* BB12 could be distinguished based on acidic stress levels (Settachaimongkon et al., 2015, 2016). This finding demonstrates that the impact of sublethal stress responses on the volatile metabolite profiles of set yogurt is species specific. Furthermore, the results indicate that several major aroma compounds, i.e., acetic acid (vinegar, pungent), 2,3-pentanedione (buttery, vanilla-like), and 2-butanone (sweet, fruity), as well as other minor carbonyl compounds, volatile organic acids, and alcohols, contribute to the separation of samples cofermented with different types of sublethally precultured probiotics (Settachaimongkon et al., 2015, 2016). On the other hand, the impact of stress-adapted probiotics on the nonvolatile polar metabolite profiles of set yogurts was not evident for all probiotic strains. A lower concentration of pyruvate and a higher concentration of acetate, formate, isoleucine, leucine, and valine were clearly observed in the samples cofermented with sublethally precultured *L. rhamnosus* GG compared to *B. animalis* ssp. *lactis* BB12 (Settachaimongkon et al., 2015). Besides this, it is documented that lactobacilli have systems for accumulating compatible solutes, i.e., glycine-betaine, carnitine, proline, and glutamate, for maintaining turgor pressure of the cell against osmotic stress (Van de Guchte et al., 2002). A lower concentration of pyruvate and proline was
clearly observed in the samples cofermented with sublethally precultured *L. plantarum* WCFS1 (Settachaimongkon et al., 2016). From a technological point of view, variations in the concentration of volatile and nonvolatile polar metabolites suggest that incorporation of sublethally precultured probiotics may influence the organoleptic quality of yogurt (Cheng, 2010; Clark, 2009). In summary, adaptive stress responses in probiotics are associated with the expression of a large number of genes, synthesis of stress–response proteins, and alteration of various physiological and functional features (Papadimitriou et al., 2015a; Sánchez et al., 2012; Van de Guchte et al., 2002). This information is important for the application of stress-adapted probiotics in yogurt since their metabolic activities may influence the biochemical and organoleptic characteristics of the fermented product (Settachaimongkon et al., 2015).

### 25.9 CONCLUDING REMARKS AND FUTURE PROSPECTS

This chapter provides information on the applications of metabolomics as an emerging strategy for the investigation of yogurt metabolite profiles. The application of NMR-based and MS-based technologies has been shown to be very successful in determining a wide range of metabolites associated with microbial activity during fermentation and storage. This information is essential, since the biochemical conversions of milk components related to microbial metabolism are responsible for the sensory characteristics of the fermented product. In addition, the combination of metabolomic-derived data with multivariate statistical analysis enables discrimination of yogurt samples according to the differences in pretreatments of base milk, types of starter cultures, and their historical background, along with the fermentation process parameters and duration of storage. Finally, metabolomics provides opportunities to assess metabolite profiles and detect potential biomarkers, which can be directly correlated with the quality, safety, and authenticity of yogurt. Future research in this field must take advantage of the development of bacterial genome projects and functional genomic technologies (Felis et al., 2015; Kowalczyk et al., 2015). A large number of publications and accessible databases generated from transcriptomic and proteomic profiling will facilitate to better understand the molecular mechanisms involved in the interaction between different lactic acid bacteria in yogurt as well as other fermented food environments. Application of genome-scale metabolic models to predict the formation of flavor compounds is one of the most relevant examples (Branco dos Santos et al., 2013; Flahaut et al., 2013; Garcia-Albornoz and Nielsen, 2013; Smid et al., 2005a,b; Teusink et al., 2006; Xu et al., 2013). This information will contribute to establish an appropriate roadmap toward improving technological and functional properties of fermented dairy products.

### ACKNOWLEDGMENTS

The previous metabolomic researches of the authors were conducted under CHE-PhD-SFR-2551 scholarship granted by the Commission on Higher Education of the Royal Thai Government. Facilities provided by Wageningen University are gratefully acknowledged. We are thankful to Dr. Kasper Hetinga, Geert Meijer, Dr. Elsa Antunes Fernandes, and Dr. Jacques Vervoort for assistance in GC/MS and NMR analysis. We also thank Bianca Hoopman, Katja Bobkova, Raoul Chin-A-Foeng, Anastasia Emelianova, and Wu Qu for technical contributions.
REFERENCES


CHAPTER 25 METABOLOMICS INVESTIGATION OF YOGURT


26.1 INTRODUCTION

Estrogens, progestogens, and androgens are sex hormones involved in the estrous cycles of mammals (Socas-Rodríguez et al., 2013). These molecules, together with mineralocorticoids and glucocorticoids (which are adrenocortical hormones), are the five main types of steroid hormones. Such hormones are derived from cholesterol, which consists of three hexagonal carbon rings and one pentagonal ring with a side chain at position 17 and two methyl groups at positions 18 and 19 (Fig. 26.1A) (Tillet and Jewels, 2005). The basic structure of cyclopentaperhydro[a]phenanthrene, also called gonane (Fig. 26.1B), can present different substituents and ring contractions or expansions. Progestogens and corticosteroids result from the partial removal of the side chain in position 17 (pregnane series of compounds; Fig. 26.1C); the total elimination of the mentioned side chain generates the androgens (androstan series; Fig. 26.1D), while the removal of the methyl group at position 19 by aromatization (with a conversion of the first hexagonal structure to a phenolic one) produces the estranes (Fig. 26.1E). Estrogens belong to this last group of compounds.

Most steroids are flat molecules with functional groups or substituents oriented in axial or equatorial positions, producing an important number of possible stereoisomers. This fact is particularly important since stereoisomerism determines the biological activity of steroids (Noppe et al., 2008). Despite their relatively simple structure, steroid hormones, which are lipophilic and have a relatively low molecular weight, can present different biologically active forms since they are highly modified in their target tissues.

Natural estrogens, also known as C_{18}-steroids, are named for their importance in the estrous cycle (physiologic changes in the reproductive system of most mammalian females). These hormones are synthesized in the ovaries, adipose tissues, and adrenal glands from androgen precursors by the action of enzymes. Apart from their primary function, which is related to the development of the female reproductive system and secondary sexual characteristics, estrogens have a crucial role in other different biological functions such as the metabolization of proteins, sugars, fats, cholesterol and minerals, some kidney functions, blood coagulation, digestion, etc. (Noppe et al., 2008). Moreover, estrogens (in combination with gestagens) directly influence the menstrual cycle. In fact, estrogen therapies are currently used to control menopause disorders.

Apart from natural estrogens, the so-called phytoestrogens also deserve a special mention. Phytoestrogens are nonsteroid molecules found in vegetables (Jarošová et al., 2015; Whitten and Patisaul, 2001). Because of their chemical similarity (Fig. 26.2), they can either act in animals in the
FIGURE 26.1
Structures of cholesterol and steroid hormones: (A) cholesterol; (B) gonane, basic structure of steroid hormones; (C) pregnane, basic structure of pregnanes and corticosteroids; (D) androstane, parent compound of androgens; (E) estrane, parent compound of estranes.

FIGURE 26.2
Structures of estrogens, mycoestrogens, and phytoestrogens: (A) estradiol (representative of estrogens); (B) zearalenone (representative of mycoestrogens); (C) isoflavones; (D) enterolactone (representative of lignanes); and (E) coumestane (representative of coumestans).
same way as natural estrogens or also produce an antiestrogenic effect (Whitten and Patisaul, 2001). Their principal function in plants is related to the protection against pathogens and herbivores although they also participate in flower coloration (Jarošová et al., 2015).

Another important group of compounds that has shown estrogenic activity and has also caught scientists’ attention are the mycotoxins of the zearalenone (ZEN) family (Fig. 26.2B). As it is well known, mycotoxins are metabolites produced by fungi that contaminate cereal grains, animal feed sources, corn silage, and hay (Jarošová et al., 2015; Pittet, 1998). Generally speaking, they all have different action mechanisms but the only ones capable of producing estrogenic effects are those of the ZEN family. These substances consist of a partially substituted β-resorcylic acid scaffold linked to a 12- or 14-membered macrolactone moiety.

The evaluation of the content of the estrogenic compounds mentioned earlier in yogurt is a topic of special concern because of the possible natural presence of most of these compounds in milk (Socas-Rodríguez et al., 2013). Dairy animals produce a large amount of milk (more than is needed for their progeny) because of targeted selection. Frequently, this production is collected in the pregnancy period with levels of natural estrogens nearly 20 times higher than the normal ones (Xu et al., 2011). Thus estrogens could transfer to milk. This problem could be intensified because it seems that pasteurization cannot inactivate estrogen activity, as some authors suggest (Qin et al., 2004). Moreover, since certain types of estrogens have an anabolic effect, some of them have also been used for this purpose. Despite the fact that animal fattening is forbidden in most countries, illegal practices could result in the presence of these kinds of molecules in milk and consequently in yogurt (Noppe et al., 2008).

Regarding phytoestrogens, it should also be remarked that soy products (including the so-called “soy milk” and “soy yogurt”) have gained importance in recent years because of their potential benefits. Such effects are claimed to be related to their high concentration of functional ingredients, with phytoestrogens very popular among them. Besides, the use of vegetable flours (which may contain large amounts of phytoestrogens and/or mycoestrogens from fungi contamination) to feed the livestock can result in the presence of these molecules in dairy products as well.

Additionally, some mycoestrogens have also been used to improve feed conversion efficiency and to promote growth rates in livestock (Metzler, 1989). Moreover, specific mycoestrogens can be produced endogenously in ruminants that have ingested ZEN (Kennedy et al., 1998). Consequently, mycoestrogens could be transferred to milk as well.

Concerning the foregoing, it is doubtless that the presence of these compounds in yogurt and, in general, in dairy products is an important issue. This is why this chapter intends to provide a global vision of the different estrogenic compounds (either with a natural or an exogenous origin) that may appear in yogurt samples, their occurrence, as well as the different analytical methodologies that have been developed and applied for their determination.
relationship with poor semen quality should be highlighted (Afeiche et al., 2013), as well as menarche at young age, late menopause, null parity, polycystic ovaries, uterine fibroids (Ganmaa and Sato, 2005; Gibson and Saunders, 2014), increase in DNA replication errors, increase in the mitotic activity of the endometrial cells (Farlow et al., 2009), and, what is specially alarming, their possible relationship with the development of breast (Hu et al., 2012), ovary, uterus, prostate, and testicle cancer (Ganmaa and Sato, 2005; Su et al., 2011).

Depending on their origin, estrogens can be classified as natural, if they are organism synthetized, or exoestrogens, which have a foreign origin.

Among natural estrogens, the most relevant, taking into account their estrogenic activity, are estradiol (E\(_2\)) and estrone (E\(_1\)), which are secreted directly by the ovaries, as well as estriol (E\(_3\)), which is an oxidation product of both of them. Apart from these free species, it is also possible to find a large number of metabolites such as, for example, sulfated, gluconated, methylated, and hydroxylated forms, which have a high importance (Farlow et al., 2009).

Among the existing natural estrogens, 17\(\beta\)-E\(_2\) presents the highest estrogenic activity, being approximately 80 times more than that of E\(_3\) and 12 times more than that of E\(_1\) (Olea Serrano et al., 2001). However, their implication in the development of cancer when they are ingested is controversial since while E\(_1\) and 17\(\beta\)-E\(_2\) appear to be the strongest carcinogens, E\(_3\) may have protective properties (Malekinejad et al., 2006; Shi et al., 2011). Apart from that, some metabolites also have estrogenic activity. Particularly, methylated and hydroxylated forms appear to be involved in hormonal disorders and cancer development (Farlow et al., 2009). In the case of sulfated and gluconated forms, although they are not active, they can be transformed into their free active forms by the bacterial sulfatases and glucuronidases present in the human gut when they are consumed (Socas-Rodríguez et al., 2013).

Regarding exoestrogens, they should be classified in two different groups if they have a natural or synthetic origin.

In the first group, it is noteworthy to mention the mycoestrogens of the ZEN family as well as phytoestrogens. In the first case, ZEN and their derivatives, zearalanone (ZAN), \(\alpha\)-zearalanol (\(\alpha\)-ZAL), \(\beta\)-zearalanol (\(\beta\)-ZAL), \(\alpha\)-zearalenol (\(\alpha\)-ZEL), and \(\beta\)-zearalenol (\(\beta\)-ZEL), have an important estrogenic activity. As a result, they can produce important disorders in females such as hyperestrogenism and infertility among other reproductive diseases including the development of hormone-dependent tumors, which has been widely demonstrated in the literature with several in vivo studies (Belhassen et al., 2015; Socas-Rodríguez et al., 2016; Xu et al., 2016). This fact is especially remarkable in the case of \(\alpha\)-ZEL and \(\alpha\)-ZAL, which are obtained as a consequence of the metabolization of the ingested ZEN in liver, stomach, and intestine, and present similar estrogenic activity to that of 17\(\beta\)-E\(_2\) (Othmen et al., 2008; Meucci et al., 2011). Besides, the negative influence of these kinds of estrogenic analytes in males has also been reported. In this sense, Benzoni et al. (2008) studied their effect on swine sperm quality by in vitro assessments, which showed an important decrease in the mobility and viability of the semen in the presence of mycoestrogens, principally in the case of ZEN, which appears to produce the highest adverse consequences.

Concerning phytoestrogens, most of them can be found in plants such as the isoflavones biochanin A, daidzein, formononetin, genistein, glycitein, and the coumestan coumestrol, or the lignan metabolites equol, enterolactone, or enterodiol, which are obtained from the metabolization of plants carried out by animals (Antignac et al., 2003, 2004; Kalač, 2013).

As regards the estrogenic activity of phytoestrogens, the controversy is considerably much greater than that of the previously mentioned compounds because of the two opposite points of view that can
be found in the literature. On the one hand, various authors defend the hypothesis that several common features of phytoestrogens with E₂ allow them to link to estrogenic receptors of the reproductive organs and also those of bones, liver, heart, and brain. This fact confers on them a preventive action against many different hormone-dependent diseases including osteoporosis, a high level of cholesterol in blood, and hypertension. Concerning hypertension, several studies have pointed to the antagonistic role of certain phytoestrogens to endogenous estrogens as the main cause of this beneficial effect. In the particular case of endometrial cancer, one of the most common causes is an overexposure of estrogens (Horn-Ross et al., 2003). In this case, phytoestrogens could block excess estrogenic activity avoiding the development of uterus cancer (Fayed, 2015). On the other hand, several studies defend an endocrine disruptor function of phytoestrogens and thus the production of several diseases related to hormonal balance in humans. This is based on the fact that, although they appear in nature under their conjugated forms, they are metabolized in the intestine of animals and are therefore transferred to the blood as active substances (Daems et al., 2016). It should be stated that they have been implicated as the cause of the development of deformities in ovaries, uterus and oviduct, premature puberty, and irregular menstrual cycles in adulthood (Fayed, 2015; Patisaul and Jefferson, 2010). However, the most alarming effect of phytoestrogens is their relationship with the development of breast cancer. This is an unsettled issue since evidence of both their beneficial and negative effects has been reported (Dewi et al., 2016; Fayed, 2015). Despite this controversy, several authors propose a clear mechanism of their role in this kind of disease deduced from in vitro studies in which phytoestrogens appear to have a stimulating action in the growth of positive estrogenic receptors of breast cancer cells. The effect can be so powerful that the administration of renowned drugs such as tamoxifen, applied to avoid this growth, can be inhibited because of the presence of phytoestrogens (Fayed, 2015).

In the second group of exoestrogens, a wide range of substances such as pesticides, polychlorinated biphenyls, phthalates, and bisphenols can be included. Although they do not have the exact same structure of natural estrogens, they can act as endocrine disruptors mimicking their activity.

Important groups of exoestrogens with a synthetic origin are the stilbenes (i.e., dienestrol, diethylstilbestrol, and hexestrol), which can mimic natural estrogen functions because of their similar structure in terms of the spatial distribution of their functional groups. This is why they have been applied in veterinary medicine to solve estrogen deficiency and also as growth promoters, despite their use being forbidden in most countries (Noppe et al., 2008).

Another synthetic compound also used as a growth promoter is 17α-ethynylestradiol, which has a structure very similar to that of natural estrogens. In addition to having a high estrogenic potential among all synthetic estrogens (Sridevi et al., 2015), this substance presents a long half-life, an aspect that has allowed its wide use as an oral contraceptive (Kuch and Ballschmiter, 2001).

Taking into account the existence of a wide variety of compounds with estrogenic activity that may also appear in yogurt samples, in this chapter the presence of natural, myco-, and phytoestrogens will only be discussed as a result of their higher occurrence.

26.3 ESTROGEN ANALYSIS

One of the objectives of any analytical method is to achieve a high sensitivity and selectivity for the feasible identification and/or quantification of the target compounds in real samples at low levels. This fact becomes particularly important in the analysis of estrogens in animal samples, and
thus in milk dairy products. The method should not only allow the determination of such compounds in these matrices at their extremely low levels (μg/L or ng/L) (Socas-Rodríguez et al., 2013) but also cover the legislative limits and permit the determination of banned, forbidden, or unknown compounds. Moreover, the selected analytical method should be easy to implement avoiding matrix interferences, and diminishing the consumption of solvents, reagents, and samples.

Despite the considerable interest in the analysis of animal estrogens (Socas-Rodríguez et al., 2013), mycoestrogens (Socas-Rodríguez et al., 2013), and phytoestrogens (Daems et al., 2016) in milk and its derivatives, the number of applications devoted to the determination of such compounds in yogurt is still scarce (Table 26.1). Moreover, the majority of published manuscripts have focused on the analysis of phytoestrogens [mainly isoflavones in soy products (Cho et al., 2013; Chun et al., 2008; Feng et al., 2008; Pyo and Song, 2009; Wang and Murphy, 1994; Wiseman et al., 2002)] because of their antioxidant activity and their possible use as alternative therapies for a range of hormone-dependent conditions (Ko, 2014; Mortensen et al., 2009; Vitale et al., 2013). Frequently, analyses are not only centered on the determination of the amount of isoflavones but also on the evolution of such amounts during the fermentation process (Cho et al., 2013; Chun et al., 2008; Feng et al., 2008; Fu and Zhang, 2013; Pham and Shah, 2009; Pyo and Song, 2009; Rossi et al., 2004).

In this sense, it has been demonstrated that 80%–90% of total estrogens are present as conjugated metabolites (Socas-Rodríguez et al., 2013) in food samples. Since conjugation could occur in different functional groups of the estrogenic molecule, diverse conjugated forms can exist for the same compound. To tackle the determination of conjugated estrogens, two different strategies have been applied (Liu et al., 2015): their indirect analysis by their hydrolytic release during the extraction step (Knight et al., 1998; Hartmann et al., 1998) and the direct assessment of these conjugates (Cavaliere et al., 2015; Cho et al., 2013; Pham and Shah, 2009; Wang and Murphy, 1994; Wiseman et al., 2002).

Historically, estrogens have been mainly analyzed in different matrices by indirect methods (Liu et al., 2015). Regarding yogurts, hydrolytic liberation requires an enzymatic digestion at 37°C using glucuronidase (to catalyze the hydrolytic cleavage of glucosidic bonds) and/or sulfatase (to catalyze the hydrolysis of sulfate esters) enzymes. The pH of the solution for enzymatic hydrolysis is often set (not only for yogurts but also for other matrices) at the optimal pH for glucuronidase or sulfatase activity (4.0–6.5). However, several disadvantages are frequently associated with enzymatic hydrolysis: the relatively high cost of enzymes, tedious optimization of the incubation temperature, pH, and time, and the existence of a high number of enzyme activity inhibitors (such as gluconic and saccharic acids). Furthermore, the enzymatic hydrolysis step is currently the bottleneck of the analysis of natural estrogen conjugates as a result of the current improvements of the analytical instrumentation. Although chemical deconjugation methods (alkali hydrolysis, acid hydrolysis, and acid solvolysis) have been developed for the analysis of natural estrogens in other matrices (Liu et al., 2015), such methodologies have not been applied to yogurts.

The direct analysis of conjugated varieties has involved the determination of sulfate and/or glucuronide forms of animal estrogens and malonyl and acetyl glycosides for phytoestrogens because these are the forms most commonly present in soy products (Pham and Shah, 2009). This direct analysis is highly desirable because the exact conjugates are monitored. However, such a method can be impractical because of the possible high number of unknown conjugated forms, while separation could be extremely difficult and standard references may not be available. It is worth mentioning that, despite the importance of the conjugation phenomenon, several articles have not addressed this problem and
<table>
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<th>Estrogens</th>
<th>Type of Yogurt (Amount)</th>
<th>Extraction Method</th>
<th>Determination Technique</th>
<th>LODs&lt;sup&gt;b&lt;/sup&gt;/LOQs&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Recoveries (%)</th>
<th>Comments</th>
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<tr>
<td>E&lt;sub&gt;2&lt;/sub&gt;, 17-β-E&lt;sub&gt;2&lt;/sub&gt;, 17α-E&lt;sub&gt;2&lt;/sub&gt;, 2-MeOE&lt;sub&gt;2&lt;/sub&gt;, E&lt;sub&gt;1&lt;/sub&gt;, β-ZAL, α-ZAL, β-ZEL, α-ZEL, ZEN (animal estrogens and mycoestrogens)</td>
<td>Whole cow liquid yogurt (2 mL)</td>
<td>Deproteination with 4 mL ACN + 100 μL HOAc. Defatting with hexane (2 mL). DLLME with CHCl&lt;sub&gt;3&lt;/sub&gt;.</td>
<td>MEKC-MS</td>
<td>5–140 μg/L&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>85–114&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Analyzed with one synthetic estrogen (EE&lt;sub&gt;2&lt;/sub&gt;). Whole cow’s, skimmed cow’s, and semiskimmed goat’s milk were also analyzed.</td>
<td>D’Orazio et al. (2015)</td>
</tr>
<tr>
<td>E&lt;sub&gt;1&lt;/sub&gt;, 17-β-E&lt;sub&gt;2&lt;/sub&gt;, 17α-E&lt;sub&gt;2&lt;/sub&gt;, 2-β-ZEL-3S-17S, 17β-E&lt;sub&gt;2&lt;/sub&gt;-3G, E&lt;sub&gt;1&lt;/sub&gt;-3G, 17β-E&lt;sub&gt;2&lt;/sub&gt;-17G, 17β-E&lt;sub&gt;2&lt;/sub&gt;-2S, E&lt;sub&gt;1&lt;/sub&gt;-17S, E&lt;sub&gt;1&lt;/sub&gt;-3S (animal estrogens and conjugated)</td>
<td>Whole milk yogurt (1 g)</td>
<td>Deproteination with 5 mL MeOH/water (80:20, v/v) containing 1% TFA. SPE (Carbograph-4).</td>
<td>UHPLC-MS/MS/MS</td>
<td>0.3–1.8 μg/kg&lt;sup&gt;b&lt;/sup&gt;, 0.9–5.5 μg/kg&lt;sup&gt;c&lt;/sup&gt;</td>
<td>86–118&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Unsalted butter and cheese were also analyzed.</td>
<td>Cavaliere et al. (2015)</td>
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<td>β-ZAL, α-ZAL, ZEL, α-ZEL, ZEN, ZAN (mycoestrogens)</td>
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<td>Extraction with 20 mL ACN/EtOAc/HOAc (49.5:49.5:1, v/v/v) (shaking 15 min, ultrasound 15 min) (×2). Freeze defatting (20 min–80°C). SPE (Oasis HLB). Defatting with hexane (3 mL).</td>
<td>HPLC-MS/MS</td>
<td>0.3 μg/kg&lt;sup&gt;b&lt;/sup&gt;, 1 μg/kg&lt;sup&gt;c&lt;/sup&gt;</td>
<td>80–94&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Analyzed with 9 antibiotics, 5 agonists, 2 sedatives, 2 pesticides, 10 mycotoxins, 5 steroid hormones (methyltestosterone, trenbolone, testosterone propionate, nandrolone phenylpropionate, megestrol acetate), and 1 synthetic estrogen (estradiol benzoate). Whole milk, powder milk, and cheese were also analyzed.</td>
<td>Xie et al. (2015)</td>
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<tr>
<td>E&lt;sub&gt;2&lt;/sub&gt;, 17-β-E&lt;sub&gt;2&lt;/sub&gt;, 17α-E&lt;sub&gt;2&lt;/sub&gt;, E&lt;sub&gt;1&lt;/sub&gt;, 2-OHE&lt;sub&gt;2&lt;/sub&gt; (animal estrogens)</td>
<td>Whole and skimmed yogurt (3 g)</td>
<td>Deproteination with 9 mL ACN + 150 μL HOAc. HF-LPME with 1-octanol.</td>
<td>HPLC-DAD/ DAD: (215 nm) (FD: 280/310 nm)</td>
<td>0.29–35.5 μg/kg&lt;sup&gt;b&lt;/sup&gt;, 0.96–118 μg/kg&lt;sup&gt;c&lt;/sup&gt;</td>
<td>82–118&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Analyzed with 4 synthetic estrogens (EE&lt;sub&gt;2&lt;/sub&gt;, DES, DS, HEX). Probiotic liquid product and white cheese were also analyzed.</td>
<td>Socas-Rodriguez et al. (2014)</td>
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<td>Daidzin, glycitin, M-daidzin, M-genistin, A-daidzin, A-glycitin, A-genistin, daidzein, glycitin, genistein (phytoestrogens and their malonyl and acetyl glycosides)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Soy yogurt (0.5 g)</td>
<td>Extraction with 5 mL 80% EtOH (2 h 60°C, stirring).</td>
<td>HPLC-DAD</td>
<td>–</td>
<td>–</td>
<td>Analyzed with glyceollins (antioestrogenic compounds). Yogurt prepared from regular soybean, soybean germinated for 3 days at 25°C, and soybean germinated under fungal action. Study of the evolution of content.</td>
<td>Cho et al. (2013)</td>
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<td>Daidzin, genistin, daidzein, genistein (phytoestrogens)</td>
<td>Chickpea yogurt (1 g)</td>
<td>Defatting with ether.</td>
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<td>–</td>
<td>–</td>
<td>Study of the changes in content with different fermentation bacteria.</td>
<td>Fu and Zhang (2013)</td>
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<td>ZEN (mycoestrogen)</td>
<td>Baby yogurt (0.5 g)</td>
<td>Extraction with 5 mL ACN/water (50:50 v/v) (shaking 10 min).</td>
<td>HPLC-MS/MS</td>
<td>–</td>
<td>103–107&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Analyzed with other 11 mycotoxins. Milk, milk-based infant formula, and milk powder were also analyzed.</td>
<td>Zhang et al. (2013)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Estrogens and phytoestrogens from soybeans and chickpeas.

<sup>b</sup> LOD: Limit of Detection.

<sup>c</sup> LOQ: Limit of Quantitation.

<sup>d</sup> Percentages are given as means.
<table>
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<tr>
<th>Estrogens</th>
<th>Type of Yogurt (Amount)</th>
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<th>Determination Technique</th>
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<td>17β-E₂, E₃ (animal estrogens)</td>
<td>Yogurt (500 g)</td>
<td>Extraction and deproteination with 150 mL MeOH: acetone (1:1, v/v) (vortex 2 min, ultrasound 5 min) (×2). MISPE.</td>
<td>HPLC-UV (202, 240 nm)</td>
<td>0.015–0.025 μmol/kg, 0.05–0.08 μmol/kg</td>
<td>72–79</td>
<td>Analyzed with progesterone, boldenone, testosterone, and penconazole. Whole milk, beef, pork, and chicken meat were also analyzed.</td>
<td>Shi et al. (2011)</td>
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<td>Daidzein, genistein, mevinolin, daidzin, genistin, glycitin, glycine (phytoestrogens)</td>
<td>Soy yogurt (0.5 g)</td>
<td>Extraction with 10 mL 80% EtOH (30 min, ultrasound).</td>
<td>HPLC-UV (202 nm)</td>
<td>–</td>
<td>–</td>
<td>Evaluation of contents of isoflavones using different fermentation bacteria.</td>
<td>Pyo and Song (2009)</td>
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<td>Genistein, daidzein, glycitein, daidzin, genistin (phytoestrogens and their malonyl and acetyl glycosides)</td>
<td>Yogurt (1 g)</td>
<td>Extraction with 5 mL 80% MeOH (2 h 60°C, stirring).</td>
<td>HPLC-DAD (260 nm)</td>
<td>10 μg/mL</td>
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<td>The amount of isoflavone produced in fermentation of yogurt supplemented with protein isolate was studied.</td>
<td>Pham and Shah (2009)</td>
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<tr>
<td>Daidzin, genistein, daidzein, genistein (phytoestrogens)</td>
<td>Black and yellow soy milk yogurts (0.5 g)</td>
<td>Extraction with 5 mL 80% MeOH (2 h 60°C, stirring).</td>
<td>HPLC-DAD (259 nm)</td>
<td>–</td>
<td>–</td>
<td>Sensory properties of yogurt were investigated after fermentation.</td>
<td>Chun et al. (2008)</td>
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<tr>
<td>Daidzin, daidzin, M-daidzin, genistein, M-genistin, glyceollin (phytoestrogens and their malonyl glycosides)</td>
<td>Soy yogurt</td>
<td>Extraction with 80% EtOH (1 h 50°C, stirring).</td>
<td>HPLC-DAD (200–400 nm)</td>
<td>–</td>
<td>–</td>
<td>Study of the fermentation process.</td>
<td>Feng et al. (2008)</td>
</tr>
<tr>
<td>Secoisolariciresinol diglucoside, secoisolariciresinol, enterodiol, enterolactone (phytoestrogens)</td>
<td>Yogurt (1 g)</td>
<td>Extraction with 20 mL MeOH (15 min, stirring) (×2).</td>
<td>HPLC</td>
<td>–</td>
<td>–</td>
<td>High-temperature pasteurized and fermented milk, milk renneting, cheese, and whey-based drinks were also analyzed.</td>
<td>Hyvärinen et al. (2006)</td>
</tr>
<tr>
<td>Daidzein, daidzin, genestein, genistin, glycitin, glycine (phytoestrogens)</td>
<td>Soy yogurt (0.5 g)</td>
<td>Defatting with hexane (10 h, Soxhlet).</td>
<td>HPLC-DAD (262 nm)</td>
<td>–</td>
<td>–</td>
<td>Study of content in samples during production process (soy beans with and without shell, cooked soy beans, and residues).</td>
<td>Rossi et al. (2004)</td>
</tr>
<tr>
<td>Daidzin, daidzin, genistin, genistein, glycitin, glycine, M-daidzin, A-daidzin, M-genistin, A-genistin, M-glycitin, A-glycitin (phytoestrogens and their malonyl and acetyl glycosides)</td>
<td>Soy yogurt (vanilla and cherry flavor) (0.5 g)</td>
<td>Extraction with 5 mL 80% EtOH (1 h 60°C, stirring). Defatting with hexane (4 × 20 mL).</td>
<td>HPLC-MS</td>
<td>0.1 mg/kg</td>
<td>–</td>
<td>Different soy food samples were also analyzed (spaghetti, meat stews, soybeans, soy milk puddings, soy milk, soy sausages, soy burgers, pastries, and bread).</td>
<td>Wiseman et al. (2002)</td>
</tr>
<tr>
<td>Genistein, daidzein, biochanin A, formononetin, equol (phytoestrogens)*a</td>
<td>Casein-based infant yogurt (2 g)</td>
<td>Enzymatic digestion (glucosidase 0.4 U/mL 24 h 37°C). Extraction with EtOH.</td>
<td>HPLC</td>
<td>54–94 ng/L*</td>
<td>–</td>
<td>Soy beverages, bovine milk, and infant formula were also analyzed.</td>
<td>Knight et al. (1998)</td>
</tr>
<tr>
<td>E&lt;sub&gt;1&lt;/sub&gt;, 17α-E&lt;sub&gt;2&lt;/sub&gt;, 17β-E&lt;sub&gt;2&lt;/sub&gt;, E&lt;sub&gt;3&lt;/sub&gt; (animal estrogens)</td>
<td>Yogurt (50 g)</td>
<td>Enzymatic digestion (25 mL 0.04 M β-glucuronidase/arylsulfatase in acetate buffer pH 5.1–5.3 overnight 37°C). Extraction with 90 mL MeOH. Defatting with hexane (2 × 40 mL). Extraction with 70, 40, and 30 mL of DCM. SPE clean-up (Amberlite XAD-2 + Celite/KOH+Al&lt;sub&gt;2&lt;/sub&gt;O&lt;sub&gt;3&lt;/sub&gt;).</td>
<td>GC-MS</td>
<td>0.01–0.3 μg/kg*</td>
<td>–</td>
<td>Analyzed with other steroid hormones (α-androsterone, 5α-dihydrotestosterone, dehydroepiandrosterone, testosterone, androstenedione, pregnenolone, progesterone, 17α-hydroxyprogesterone). Beef, veal, pork, poultry, eggs, fish, plants, yeast, milk, cream, butter, cheese, and alcoholic beverage samples were also analyzed. Steroids were derivatized with MSTFA/TMIS/DTE.</td>
<td>Hartmann et al. (1998)</td>
</tr>
<tr>
<td>Daidzein, genistein, glycitein, daidzin, genistin, glycinin, A-daidzin, A-genistin, A-glycitin, M-daidzin, M-genistin, M-glycitin (phytoestrogens and their malonyl and acetyl glycosides)*a</td>
<td>Tofu yogurt (2 g)</td>
<td>Deproteination and extraction with 10 mL of ACN + 2 mL 0.1 N HCl (2 h, stirring).</td>
<td>HPLC-DAD (254 nm)</td>
<td>100–185 μg/L*</td>
<td>70–95</td>
<td>Soy beans, soy flour, instant soy beverage powder, tofu, tempeh, bean paste, miso, fermented bean curd, soy hot dog, soy bacon, tempeh burger, soy cheese, and soy noodles were also analyzed.</td>
<td>Wang and Murphy (1994)</td>
</tr>
</tbody>
</table>

| ACN, Acetonitrile; DAD, diode array detection; DCM, dichloromethane; DES, diethylstilbestrol; DLLME, dispersive liquid–liquid microextraction; DS, dienestrol; DTE, dithioerythritol; EE<sub>2</sub>, 17α-ethynylestradiol; 17α-E<sub>2</sub>, 17α-estradiol; 17β-E<sub>2</sub>, 17β-estradiol; 17β-E<sub>2</sub>-3G, 17β-estradiol-3-glucuronide; 17β-E<sub>2</sub>-17G, 17β-estradiol-17-glucuronide; 17β-E<sub>2</sub>-3G-17S, 17β-estradiol-3-glucuronide-17β-sulfate; 17β-E<sub>2</sub>-3S-17S, 17β-estradiol-3,17β-disulfate; 17β-E<sub>2</sub>-3S, 17β-estradiol-3-sulfate; 17β-E<sub>2</sub>-17S, 17β-estradiol-17-sulfate; E<sub>1</sub>, estradiol; E<sub>1</sub>-3S, estradiol-3-sulfate; E<sub>1</sub>, estrone; E<sub>1</sub>-3G, estrone-3-glucuronide; E<sub>1</sub>-3S, estrone-3-sulfate; E<sub>1</sub>-17S, estrone-17-sulfate; EtOH, ethanol; FD, fluorescence detection; HEX, hexestrol; HF, hollow fiber; HOAc, acetic acid; HPLC, high-performance liquid chromatography; LPME, liquid phase microextraction; MEKC, micellar electrokinetic chromatography; 2-MeOE<sub>2</sub>, 2 methoxyestradiol; MeOH, methanol; MISPE, molecularly imprinted solid-phase extraction; MS, mass spectrometry; MS/MS, tandem mass spectrometry; MSTFA, N-methyl-N-trimethylsilyltrifluoroacetamide; 2-OHE<sub>2</sub>, 2-hydroxyestradiol; SPE, solid-phase extraction; TFA, trifluoroacetic acid; TMIS, trimethylisobutylsilane; UHPLC, ultra-high-performance liquid chromatography; ZAN, zearalanone; ZEN, zearalenone; α-ZAL, α-zearalanol; β-ZAL, β-zearalanol; α-ZEL, α-zearalenol; β-ZEL, β-zearalenol.

*Malonyl glycosides (M-) and acetyl glycosides (A-).

*Limits of detection.
*Limit of quantification.
Expressed as accuracy.
have been limited to the analysis of free forms (Chun et al., 2008; Fu and Zhang, 2013; Hartmann et al., 1998; Hyvärinen et al., 2006; Knight et al., 1998; Pyo and Song, 2009; Rossi et al., 2004; Shi et al., 2011; Socas-Rodríguez et al., 2014).

Regarding extraction methods, natural estrogens are lipophilic compounds and tend to accumulate in fats. Sometimes the amount of coextracted lipids could be particularly high, especially for nonvolatile ones, which in some cases can damage the analytical instrumentation. Besides, food samples (and among them yogurt matrices) contain a high proportion of proteins. Such proteins can produce important matrix effects (suppression or increase in the detectors’ signal) and may cause contamination and blockage or irreversible damage/adsorption onto the stationary phases. For this reason, authors frequently include a pre- or postdeproteination (Cavaliere et al., 2015; D’Orazio et al., 2015; Pham and Shah, 2009; Shi et al., 2011; Socas-Rodríguez et al., 2014; Wang and Murphy, 1994) and/or defatting (D’Orazio et al., 2015; Fu and Zhang, 2013; Hartmann et al., 1998; Wiseman et al., 2002; Xie et al., 2015) step in addition to the main extraction procedure. In general, precipitation of proteins can be carried out by the addition of organic solvents, acids, salts, and/or metal ions (Polson et al., 2003). In the analysis of estrogenic compounds in yogurts, organic solvents have been mainly used [i.e., acetonitrile (ACN) (D’Orazio et al., 2015; Socas-Rodríguez et al., 2014; Wang and Murphy, 1994), methanol (MeOH) (Cavaliere et al., 2015; Shi et al., 2011), and acetone (Shi et al., 2011)] with or without acid addition [acetic acid (HOAc) (D’Orazio et al., 2015; Socas-Rodríguez et al., 2014), trifluoroacetic acid (TFA) (Cavaliere et al., 2015), and hydrochloric acid (HCl) (Wang and Murphy, 1994)], although some salts or metals have also been employed (Pham and Shah, 2009). The amount of these precipitating agents was directly related to that of the sample. Since it is frequent to analyze between 0.5 and 3 g of sample [although examples of methods using 50 (Hartmann et al., 1998) or 500 g (Shi et al., 2011) can be found], volumes in the range 4–10 mL were usually employed [with punctual consumptions of 150 mL (Shi et al., 2011)]. Defatting was also carried out using apolar or low polarity solvents such as hexane (D’Orazio et al., 2015; Hartmann et al., 1998; Rossi et al., 2004; Wiseman et al., 2002) or ether (Fu and Zhang, 2013; Xie et al., 2015) with volumes between 2 and 80 mL, or by the so-called freeze-out procedure (Xie et al., 2015), which consists in the precipitation of lipids at low temperature. Commonly, samples were initially freeze dried (Cho et al., 2013; Chun et al., 2008; Feng et al., 2008; Fu and Zhang, 2013; Pham and Shah, 2009; Rossi et al., 2004; Wang and Murphy, 1994; Wiseman et al., 2002) or simply frozen (Shi et al., 2011) before the extraction procedure. The majority of the extraction methods have used organic solvents to isolate these target compounds (Cho et al., 2013; Chun et al., 2008; Feng et al., 2008; Fu and Zhang, 2013; Hartmann et al., 1998; Hyvärinen et al., 2006; Knight et al., 1998; Pham and Shah, 2009; Pyo and Song, 2009; Rossi et al., 2004; Shi et al., 2011; Wang and Murphy, 1994; Wiseman et al., 2002; Xie et al., 2015; Zhang et al., 2013). Frequently, such extraction has been assisted by heating (Cho et al., 2013; Chun et al., 2008; Feng et al., 2008; Fu and Zhang, 2013; Wiseman et al., 2002) and/or by ultrasound (Shi et al., 2011; Pyo and Song, 2009; Xie et al., 2015), vortex (Pham and Shah, 2009; Shi et al., 2011), or mechanical stirring (Cho et al., 2013; Chun et al., 2008; Feng et al., 2008; Hyvärinen et al., 2006; Rossi et al., 2004; Wang and Murphy, 1994; Wiseman et al., 2002; Xie et al., 2015; Zhang et al., 2013). In general, conjugates are more polar than the free forms (Daems et al., 2016) and therefore, whether the conjugates are extracted or not, the extraction process has been frequently carried out with polar solvents such as alcohols [MeOH (Chun et al., 2008; Hartmann et al., 1998; Hyvärinen et al., 2006; Pham and Shah, 2009; Rossi et al., 2004; Shi et al., 2011) and ethanol (Cho et al., 2013; Feng et al., 2008; Fu and Zhang, 2013; Knight et al., 1998; Pyo and Song, 2009; Wiseman et al., 2002)].
or ACN (Wang and Murphy, 1994; Xie et al., 2015; Zhang et al., 2013), often mixed with different proportions of water. As previously mentioned, organic solvents can also precipitate proteins, thus deproteination and/or defatting are often accomplished at the same time as the extraction, avoiding additional sample pretreatment steps.

Because of the low levels of estrogens in yogurts, sometimes it is necessary to concentrate the extracts before the final instrumental analysis. Usually, this concentration has been made by evaporation of the extraction solvent. Moreover, this preconcentration, as well as an additional cleanup, have been executed in certain applications by means of solid-phase extraction (SPE) (Cavaliere et al., 2015; Hartmann et al., 1998; Shi et al., 2011; Xie et al., 2015), liquid-phase microextraction (LPME) [i.e., dispersive liquid–liquid microextraction (D’Orazio et al., 2015), and hollow-fiber liquid-phase microextraction (HF-LPME) (Socas-Rodríguez et al., 2014)]. It is surprising that, despite the high use of SPE for the analysis of animal estrogens, mycoestrogens (Socas-Rodríguez et al., 2013), and phytoestrogens (Daems et al., 2016) in milk, only a few articles have used it for yogurt sample analysis. The reason might be the limited number of methods developed for this kind of matrix. In any case, the sorbents most commonly used (i.e., graphitized carbon, polyvinylpyrrolidone-divinylbenzene copolymer, hydrophobic crosslinked polystyrene copolymer, celite, alumina, and molecularly imprinted polymer) have also been demonstrated to be effective for the extraction of estrogens from yogurts (Cavaliere et al., 2015; Hartmann et al., 1998; Shi et al., 2011; Xie et al., 2015).

It should be pointed out that the methods based on the use of LPME are in line with one of the current trends in analytical chemistry: the use of quick, simple, and low-solvent-consuming methods. Another important aspect is the additional cleanup offered by these liquid-based microextraction techniques. As can be seen in Fig. 26.3, which represents the chromatogram obtained by Socas-Rodríguez et al. (2014) after the application of an HF-LPME procedure to the aqueous extract of several yogurt samples after deproteination, HF-LPME provides very clean extracts. This fact can be appreciated by comparing blank and spiked chromatograms in which no interferences precluding the correct quantification of natural estrogens can be observed. For these reasons and, since such methodologies have been successfully used before for the determination of these steroid hormones in food, the mentioned procedures will probably be intensely applied in the future for yogurt analysis.

Up to now, diverse methodologies have been proposed for the determination of natural estrogens (Barreiros et al., 2016; Daems et al., 2016; Socas-Rodríguez et al., 2013) in different biological, environmental, and agrofood samples. Many of these studies were based on the use of immunoassays, chromatographic techniques [gas chromatography (GC) or liquid chromatography (LC)], and electrophoretic methods. However, the analysis of such compounds in yogurt was made almost exclusively by LC. Immunoassays, despite being highly sensitive and highly specific for one compound and offering high-throughput screening at a relatively low cost, have not been used probably because of the challenge of preparing the antibodies, the cross-reactivity between similar analytes, and the complexity of the automatization of the process. Concerning GC and capillary electrophoresis (CE), these techniques were used in one occasion each. The limited number of GC applications could be explained by the fact that the low volatility of estrogens requires the introduction of an additional derivatization step to increase the volatility and stability of the target analytes. Such derivatization procedures are time consuming and can sometimes result in analyte loss. However, it is worth mentioning that in the unique work in which GC has been used to analyze yogurt samples, derivatization was carried out with N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA), trimethylsilyldisiloxane (TMIS), and dithioerythritol (DTE) (Hartmann et al., 1998). Such reagents produced a
silylation process with a potent and selective trimethylsilyl introduction mainly in OH groups, because of the association between MSTFA and TMIS as well as the additional catalytic effect of DTE. Alternatively, CE was used in the modality of micellar electrokinetic chromatography using ammonium perfluorooctanoate, a mass spectrometry (MS)-friendly surfactant (D’Orazio et al., 2015). CE could represent an interesting alternative because it offers high separation efficiency, short analysis time, low consumption of solvents, and simplicity. By contrast, the major drawbacks, and probably because of the low number of applications developed, are the low sensitivity associated with the short optical path length of the capillaries when UV–vis detection is used and the small sample volumes injected.

Since a small number of papers have been published dealing with the analysis of estrogens in yogurts using the previously mentioned techniques, a further increase in the quantity of methodologies using immunoassays, GC, and CE is expected because of the interesting results offered by these kinds of methods.
Concerning LC, separation has always been made in reversed-phase mode. For this purpose, columns made of C_{18}-silica with lengths between 50 and 250 mm, internal diameters in the range 2.1–4.6 mm, and particles of 1.9–5 μm were used. Particles with diameters lower than 2 μm have been used only on one occasion (Cavaliere et al., 2015). These kinds of particles present the advantage of using ultrahigh performance liquid chromatography. In these chromatographic systems, columns are compatible with high pressures, which decrease the analysis time up to nine times (compared to conventional high-performance liquid chromatography), providing efficiencies that can also be up to nine times higher (Guillarme et al., 2010), and reducing the consumption of organic solvents and sample volumes (Socas-Rodríguez et al., 2015). Despite its limited use, its recent implementation and the associated benefits could ensure an important number of applications in the near future. Regarding mobile phases, mixtures of polar solvents such as ACN (Cavaliere et al., 2015; Feng et al., 2008; Pham and Shah, 2009; Pyo and Song, 2009; Shi et al., 2011; Socas-Rodríguez et al., 2014; Wang and Murphy, 1994; Wiseman et al., 2002; Xie et al., 2015) or MeOH (Cho et al., 2013; Chun et al., 2008; Fu and Zhang, 2013; Hyvärinen et al., 2006; Zhang et al., 2013) with water were preferably utilized, although other solvents such as acetone have also been used (Cavaliere et al., 2015). Moreover, some additives [i.e., ammonium formate (Cavaliere et al., 2015; Xie et al., 2015; Zhang et al., 2013), formic acid (Socas-Rodríguez et al., 2014; Xie et al., 2015; Zhang et al., 2013), HOAc (Cho et al., 2013; Chun et al., 2008; Fu and Zhang, 2013; Wang and Murphy, 1994; Wiseman et al., 2002), and phosphoric acid (Hyvärinen et al., 2006; Pham and Shah, 2009; Pyo and Song, 2009)] have been employed on some occasions to improve separation or to enhance MS sensitivity. In particular, the use of acidic mobile phases enhances chromatographic resolution and peak shape because of the acid/base behavior of estrogens. The mobile phase flow rate and temperature used have depended on its composition, column type, and compounds selected. In the mentioned articles, the flow rates varied between 0.3 and 1 mL/min, the temperature was from 25 to 40°C, and the injection volume was from 5 to 20 μL. Concerning detection, MS was used both in negative (Cavaliere et al., 2015; Xie et al., 2015) (in [M−H]^− for conjugates) and positive (Wiseman et al., 2002; Xie et al., 2015) modes (in [M+H]^+ form). Concerning other detection systems, fluorescence detection has been also used (Socas-Rodríguez et al., 2014), exploiting the inherent native fluorescence of some estrogens (i.e., E_3, 17β-E_2, 17α-E_2). Nonetheless, the preferred detection mode has been without any doubt the one based on UV radiation absorption. Both UV detectors (Fu and Zhang, 2013; Pyo and Song, 2009; Shi et al., 2011) and diode array detection (DAD) (Cho et al., 2013; Chun et al., 2008; Feng et al., 2008; Pham and Shah, 2009; Socas-Rodríguez et al., 2014; Wang and Murphy, 1994) have been used for this purpose to determine these compounds in the 200–400 nm range (mainly between 200 and 260 nm). It should be noted that DAD or UV detectors have a limited selectivity despite being the preferred detection techniques in terms of economy. Thus when they are used, highly selective extraction methods should be developed to ensure a correct identification and/or quantification. Alternatively, the use of MS coupled to LC can guarantee the unambiguous detection and determination of molecules in different samples and, consequently, applications using this detector will increase in the future.

Fig. 26.4 shows a summary of the methodologies that have been applied to estrogen determination in yogurt.

Finally, it should be noted that, in some cases, natural animal, myco-, and phytoestrogens have been analyzed together with other estrogenic compounds such as synthetic estrogens (D’Orazio et al., 2015; Socas-Rodríguez et al., 2014; Xie et al., 2015), with antiestrogenic compounds (Cho et al., 2013) or steroid hormones (Hartmann et al., 1998; Shi et al., 2011; Xie et al., 2015). This combined analysis is
justified not only by the chemical similarity but also by the effect of such substances in the human organism. Sometimes, estrogens have also been analyzed together with different organic residues (such as pesticides, antibiotics, mycotoxins, and veterinary substances) (Xie et al., 2015; Zhang et al., 2013). Moreover, other samples different from yogurt but directly related with it or with the prime material [such as cow’s (D’Orazio et al., 2015; Hartmann et al., 1998; Knight et al., 1998; Shi et al., 2011; Xie et al., 2015; Zhang et al., 2013), soy (Hyvärinen et al., 2006), and goat’s milk (D’Orazio et al., 2015), butter (Cavaliere et al., 2015; Hartmann et al., 1998), cheese (Cavaliere et al., 2015; Hartmann et al., 1998; Hyvärinen et al., 2006; Socas-Rodríguez et al., 2014; Xie et al., 2015), meat (Hartmann et al., 1998; Shi et al., 2011), beverages (Hartmann et al., 1998; Hyvärinen et al., 2006), soy foods (Knight et al., 1998; Wang and Murphy, 1994; Wiseman et al., 2002), infant formula (Knight et al., 1998; Xie et al., 2015; Zhang et al., 2013), and fish (Hartmann et al., 1998)] have also been analyzed in a high percentage of these articles.

### 26.4 OCCURRENCE

As previously indicated, estrogenic compounds can appear in yogurt samples by different routes depending on the type of analyte and its origin as well as its preparation and storage (Fu and Zhang, 2013).
Contrary to what happens with other dairy products, the occurrence of natural estrogens and their metabolites in yogurt has not been so widely reported in the literature. However, there is evidence of the presence of these compounds in yogurt samples (Cavaliere et al., 2015; Hartmann et al., 1998; Shi et al., 2011), an aspect that is obviously linked with their content in the milk used as raw material.

Current farming practices frequently involve the use of milk from pregnant livestock; in fact, it is estimated that around 75% of commercial milk comes from these kinds of cows (Afeiche et al., 2013), which present a higher concentration of endogenous estrogens during this period. This is particularly important for $E_1$ and $E_2$, since they can easily transfer from blood to milk in the mammary glands because of their lipophilic character (Socas-Rodríguez et al., 2013).

Despite the presence of estrogens in these kinds of products, neither the European Union nor the United States has established maximum residue limits in milk or yogurt samples. However, as it was indicated earlier, the presence of estrogens has already been evidenced in yogurts. In particular, both $17\beta$-E$_2$ in the range 0.0214–0.113 $\mu$g/kg and $E_1$ at 0.16 $\mu$g/kg were found, while $17\alpha$-E$_2$ and E$_3$ were never detected (Hartmann et al., 1998; Shi et al., 2011). On the other hand, and although they are difficult to quantify in dairy products, it is known that 80%–90% of the content of estrogens in milk derivatives are methylated, hydroxylated, glucoronated, or sulfated metabolites. In fact, this kind of compound has already been found in yogurt by Cavaliere et al. (2015) who carried out the determination of glucuronated and sulfated forms in whole milk yogurt evidencing the presence of estrone-3-sulfate at trace levels (Cavaliere et al., 2015). This aspect is of great concern since although this species is not biologically active it can be metabolized by humans liberating its free, and consequently active, form.

While the presence of mycoestrogens has not been reported until now in yogurt samples, there are a large number of studies regarding the occurrence of phytoestrogens in soy, tofu, and animal milk yogurt samples (Knight et al., 1998; Wang and Murphy, 1994; Wiseman et al., 2002) as well as the evolution of their content during the preparation process (Cho et al., 2013; Chun et al., 2008; Feng et al., 2008; Fu and Zhang, 2013; Pham and Shah, 2009; Pyo and Song, 2009; Rossi et al., 2004). The presence of phytoestrogens in soy yogurt is caused by the transformation of the isoflavone glucosides glycitin, daidzin, and genistin, present in soy and other legumes, into their aglycones glycitein, daidzein, and genistein, which has demonstrated the behavior of soy yogurt as a preventive or enhancer of hormonal diseases and cancer development (Daems et al., 2016; Fayed, 2015). This conversion is carried out by bacteria during the fermentation process in such a way that it will increase the concentration according to the length of the procedure. This is why most articles are focused on the total content of aglycones but not on the individual occurrence of this kind of compound since the aim of these studies is the evaluation of the efficiency of such transformation.

As can be observed in Table 26.2, the data currently available is very variable, being difficult to compare because of the different fermentation conditions, which also include the use of a different type of bacteria in each case. As an example, while Chun et al. (2008) found a total concentration of genistein and daidzein higher than 1300 mg/kg after 10 h of fermentation (Chun et al., 2008), Pyo and Song (2009) only found a concentration of 566 mg/kg of the total of genistein, daidzein, and glycitein after 24 h (Pyo and Song, 2009). Other authors have also carried out the determination of these analytes individually. In this sense Cho et al. (2013) found concentrations in the ranges 417–635 mg/kg, 612–695 mg/kg, and 132–206 mg/kg for daidzein, glycitein, and genistein, respectively, after 12 h of fermentation, while Feng et al. (2008) only detected trace levels of the same analytes. Another example is the work of Fu and Zhang (2013) in which a chickpea yogurt was analyzed obtaining concentrations of 40 and 50 mg/kg for daidzein and genistein, respectively, after 4 h of processing.
Table 26.2 Occurrence of Estrogenic Compounds in Analyzed Yogurt Samples

<table>
<thead>
<tr>
<th>Analyzed Estrogens</th>
<th>Type of Yogurt (Amount)</th>
<th>Level of Estrogens Found</th>
<th>Comments</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Daidzin, glycitin, M-daidzin, M-genistin, A-daidzin, A-glycitin, A-genistin, daidzein, genistein (phytoestrogens and their malonyl and acetyl glycosides)a</td>
<td>Soy yogurt (0.5 g)</td>
<td>Daidzein (417–635 mg/kg), glycitein (612–695 mg/kg), genistein (132–206 mg/kg)</td>
<td>Yogurt prepared from regular soybean, soybean germinated for 3 days at 25°C, and soybean germinated under fungal action. Study of the evolution of content during the fermentation process in three different yogurts. Data obtained after 12 h of fermentation.</td>
<td>Cho et al. (2013)</td>
</tr>
<tr>
<td>Daidzin, genistin, daidzein, genistein (phytoestrogens)</td>
<td>Chickpea yogurt (1 g)</td>
<td>Daidzein (39.92 mg/kg) and genistein (50.34 mg/kg)</td>
<td>Study of the changes in content with different fermentation bacteria. Data obtained after 4 h of fermentation.</td>
<td>Fu and Zhang (2013)</td>
</tr>
<tr>
<td>17β-E2, E3 (animal estrogens)</td>
<td>Yogurt (500 g)</td>
<td>17β-E2 (0.0314–0.113 μg/kg)</td>
<td>Evaluation of contents of isoflavones using different fermentation bacteria and during the storage process. Data obtained after 24 h of fermentation.</td>
<td>Shi et al. (2011)</td>
</tr>
<tr>
<td>Daidzin, genistin, mevinolin, daidzin, genistin, glycitin, glycitein (phytoestrogens)</td>
<td>Soy yogurt (0.5 g)</td>
<td>Phytoestrogens (566 mg/kg)</td>
<td>–</td>
<td>Pyo and Song (2009)</td>
</tr>
<tr>
<td>Genistein, daidzein, glycitein, daidzin, glycitin, genistin, M-daidzin, M-glycitin, M-genistin, A-daidzin, A-glycitin, A-genistin (phytoestrogens and their malonyl and acetyl glycosides)a</td>
<td>Yogurt (1 g)</td>
<td>Phytoestrogens (150.10 mg/kg)</td>
<td>The amount of iso- flavone produced in the fermentation of yogurt supplemented with protein isolate was studied and their content was evaluated during the storage process.</td>
<td>Pham and Shah (2009)</td>
</tr>
<tr>
<td>Daidzin, genistin, daidzein, genistein (phytoestrogens)</td>
<td>Black and yellow soy milk yogurts (0.5 g)</td>
<td>Daidzein and genistein (&gt;1300 mg/kg)</td>
<td>Evaluation of contents of isoflavones after 10 h of fermentation.</td>
<td>Chun et al. (2008)</td>
</tr>
</tbody>
</table>
Table 26.2 Occurrence of Estrogenic Compounds in Analyzed Yogurt Samples—cont’d

<table>
<thead>
<tr>
<th>Analyzed Estrogens</th>
<th>Type of Yogurt (Amount)</th>
<th>Level of Estrogens Found</th>
<th>Comments</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Daidzein, daidzin, M-daidzin, genistein, M-genistin, glyceollin (phytoestrogens and their malonyl glycosides)(^a)</td>
<td>Soy yogurt</td>
<td>Daidzein, genistein, and glycitein were found at trace levels</td>
<td>Evaluation of contents of isoflavones after 6 h of fermentation.</td>
<td>Feng et al. (2008)</td>
</tr>
<tr>
<td>Daidzein, daidzin, genistein, genestin, glycitein, glycitin (phytoestrogens)</td>
<td>Soy yogurt (0.5 g)</td>
<td>Daidzein (0.4 mg/kg), genistein (0.9 mg/kg), and glycitein (0.4 mg/kg)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Daidzin, daidzein, genistein, genestin, glycitein, glycitin (phytoestrogens and their malonyl and acetyl glycosides)(^a)</td>
<td>Soy yogurt (vanilla and cherry flavor) (0.5 g)</td>
<td>Daidzein (10–13 mg/kg), genistein (18–20 mg/kg)</td>
<td>Study of content in samples during production process. Data obtained at the end of the process.</td>
<td>Rossi et al. (2004)</td>
</tr>
<tr>
<td>Genistein, daidzein, biochanin A, formononetin, equol (phytoestrogens)</td>
<td>Casein-based infant yogurt (2 g)</td>
<td>Daidzein (0.2–0.7 mg/L)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E(_1), 17(\alpha)-E(_2), 17(\beta)-E(_2), E(_3) (animal estrogens)</td>
<td>Yogurt (50 g)</td>
<td>17(\beta)-E(_2) (&lt;0.02 (\mu)g/kg), E(_1) (0.16 (\mu)g/kg)</td>
<td>Steroids were derivatized with MSTFA/TMIS/DTE.</td>
<td>Hartmann et al. (1998)</td>
</tr>
<tr>
<td>Daidzein, genistein, glycitein, daidzin, genistin, glycitin, A-daidzin, A-genistin, A-glycitin, M-daidzin, M-genistin, M-glycitin (phytoestrogens and their malonyl and acetyl glycosides)(^a)</td>
<td>Tofu yogurt (2 g)</td>
<td>Daidzein (57 mg/kg), genistein (94 mg/kg), glycitein (12 mg/kg)</td>
<td></td>
<td>Wang and Murphy (1994)</td>
</tr>
</tbody>
</table>

\(\text{DES, Diethylstilbestrol; DS, dienestrol; DTE, dithioerythritol; EE}_2, 17\alpha\text{-ethynylestradiol; E}_1\text{-3G, estrone-3-glucuronide; 17\(\beta\)-E}_2\text{-3G, 17\(\beta\)-estradiol-3-glucuronide; 17\(\beta\)-E}_2\text{-17G, 17\(\beta\)-estradiol-17-glucuronide; 17\(\beta\)-E}_2\text{-3G-17\(\beta\)-sulfate; 17\(\beta\)-E}_2\text{-3S, 17\(\beta\)-estradiol-3-sulfate; 17\(\beta\)-E}_2\text{-17S, 17\(\beta\)-estradiol-17-sulfate; 17\(\beta\)-E}_2\text{-3S-17S, 17\(\beta\)-estradiol-3,17\(\beta\)-disulfate; 17\(\alpha\)-E}_2\text{-17S, 17\(\alpha\)-estradiol-17-sulfate; E}_1\text{-17S, estrone-17-sulfate; HEX, hexestrol; 2-MeOE}_2, 2\text{-methoxyestradiol; MSTFA, N-methyl-N-trimethylsilyltrifluoroacetamide; 2-OHE}_2, 2\text{-hydroxyestradiol; TMIS, trimethylsilylosilane.} \)

\(^a\text{Malonyl glycosides (M-) and acetyl glycosides (A-).}\)
Apart from isoflavone, other phytoestrogens such as lignans have been analyzed since these can appear in animal milk yogurt samples as a result of the metabolism of plant lignans by the microflora of animal colon. In this sense, Hyvärinen et al. (2006) evaluated the degradation of the precursor secoisolariciresinol diglucoside into their metabolites enterodiol and enterolactone during yogurt process preparation and storage. Results showed that, contrary to what happened with isoflavone phytoestrogens, lignan precursors are very stable to fermentation and storage since no metabolites were found during the study.

26.5 CONCLUSIONS

The presence of estrogens in yogurt is a topic of special concern. Yogurt represents an important fermented derivative from milk. Because of the huge demand, animal milk is obtained even during the gestation period, what can lead to the presence of natural estrogens in milk and thus in yogurt. Phytoestrogens and mycoestrogens may also appear, though in this last case they have not been analyzed in milk samples yet. Furthermore, soy is recognized as the principal dietary source of phytoestrogens and soy-based products contain significant quantities of them. The so-called “soy yogurt” has appeared as an interesting alternative to traditional animal yogurts because of their reduced level of cholesterol and saturated fat and because they are free of lactose. Also different probiotic and dairy-supplemented products, based on soy yogurt, have been launched and commercialized because of the growing consumer demand for healthy products and natural ingredients.

Animal estrogens have a vital importance in the estrous cycle, in female sexual differentiation of mammalians, and in the participation of different biological functions. The abnormal intake of such substances is related to different disorders of the endocrine system, diseases of the reproductive system, and even cancer. Mycoestrogens act as endocrine disruptors producing reproductive illness and hormone-dependent tumors. Controversy has surrounded the activity of phytoestrogens in humans. Some authors indicated that they can be used to prevent hormone-dependent illness (including cancer), whereas others claim that they act as endocrine disruptors and produce diverse disorders (even cancer).

Regarding methodologies of analysis, several articles have proposed a wide variety of procedures. However, bearing in mind the importance of such analysis, it would be highly desirable to see further development of sensitive, quick, and inexpensive methodologies and also their application to a high number as well as a wide variety of yogurt samples since the current data available are very scarce. So far, extraction procedures have implied the treatment with polar solvents of frozen samples. Sometimes, and despite the fact that solvents produce precipitation of proteins, additional steps of deproteination and/or defatting have been introduced. Moreover, extra clean-up/preconcentration stages, by SPE or LPME, have also been introduced. Concerning the determination techniques, although GC (with analyte derivatization) and CE have been used, the vast majority of approaches have implied the utilization of LC as a separation method. DAD and UV detectors have been the preferred detection systems. It is worth mentioning that since the largest percentage of estrogens in food samples is in their conjugated form, the analysis of these conjugates has been carried out either by direct analysis of such molecules or by their indirect analysis after hydrolytic liberation. Additionally, the mentioned compounds have been usually analyzed together with other estrogenic compounds (mycotoxins or synthetic estrogens) or steroid hormones.

Estrogens can be present in yogurt at significant concentrations, since they are fatty matrices that can accumulate high concentrations of these lipophilic compounds. In this sense, animal estrogens have been found at concentrations in the range <0.02–0.16 μg/kg, whereas mycoestrogens have not been
reporting yet. Concerning phytoestrogens, the range of concentrations at which they have been found was wider (0.4–1300 mg/kg). This fact can be explained because the articles that determine these compounds have usually evaluated the changes produced during the fermentation process and the amounts found depend on such a preparation procedure. Despite these results, the European Union and the Food and Drug Administration have not established maximum residue limits in yogurts or milk yet.

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27.1 INTRODUCTION

It is well established that appropriate nutrition choices become strategic in the maintenance of a healthy body weight and in the promotion of overall health. Numerous dietary interventions have been reported to protect against chronic disease including metabolic syndrome (Andersen and Fernandez, 2013), diabetes (Salas-salvad et al., 2016), heart disease (Mayor, 2016), and others. There is some evidence that the implementation of certain dietary priorities including consumption of dairy products, with a major focus in yogurt, may contribute to healthy lifestyles associated with decreased risk of chronic disease (Mozaffarian, 2016). The association of yogurt with healthy outcomes and general well-being has been known for many years. Recently a scoping review reported that 213 studies have been conducted to determine yogurt health benefits (Glanville et al., 2015) on different aspects of health including bone health, weight management, gastrointestinal health, diabetes, and Parkinson disease to mention just a few. This review confirms for the most part the protective effects of yogurt against different conditions as well as its role in health (Glanville et al., 2015).

To further support the role of dairy products in health, it has been reported that if Americans consumed at least 3–4 servings/day of dairy products, the 5-year cumulative savings for health care benefits would be over $200 billion (McCarron and Heaney, 2004). In addition, public health and health economic analysis support the recommendation that the preferred source of calcium for older men and women is dairy products (Ethgen et al., 2015). However, analysis of epidemiological data from the National Health and Nutrition Examination Survey (Mangano et al., 2011) and other sources (Villegas et al., 2010) conclude that Americans consume 70% of recommended calcium intake. Since consumption of dairy products is associated, as mentioned earlier, with decreased risk of a variety of metabolic diseases, the importance of dairy consumption needs to be emphasized.

Health effects of yogurt have not been extensively studied. There are numerous benefits in the components of yogurt, which include large quantities of important nutrients in combination with low calories (Glanville et al., 2015). In addition, the beneficial effects of lactic acid bacteria present in yogurt in improving gastrointestinal conditions including constipation, diarrheal diseases, colon cancer, inflammatory bowel disease, and others have been studied (Adolfsson et al., 2004). For the purposes of this chapter, the focus will be on the protective effects of yogurt on (1) metabolic syndrome, (2) heart disease, and (3) diabetes. In the next section, evidence derived from epidemiological studies, clinical interventions as well as mechanistic effects of major nutrients from dairy/yogurt on blood pressure, body weight, plasma glucose, and plasma lipids will be addressed.
27.2 EPIDEMIOLOGICAL STUDIES

27.2.1 METABOLIC SYNDROME

Metabolic syndrome (MetS) consists of a constellation of indicators including central obesity, dyslipidemia, hypertension, and hyperglycemia, known to increase the risk for diabetes by fivefold and the risk for heart disease by twofold (Grundy, 2007). Thus dietary interventions that might decrease the development of MetS become very important. Based on the inverse associations reported between calcium (where dairy products are major source) intake and hypertension and glucose intolerance, it has been postulated that calcium deficiency influences the risk of developing metabolic syndrome (Major et al., 2008). The association between dairy consumption and metabolic syndrome has been examined in several cohort studies. For example, the evaluation of a cohort of 16,000 adults from the CARDIA study demonstrated that the highest quintile of dairy consumption decreased the risk of metabolic syndrome by 13% compared to the lowest quintile (Pereira et al., 2002). Similarly, in the DESIR study a higher consumption of dairy products and calcium was associated with a lower 9-year incidence of metabolic syndrome (Fumeron et al., 2011). In contrast to these findings, data from the British Women’s Heart and Health study reported that abstaining from milk reduced the odds for developing MetS by 45% (Lawlor et al., 2005). Further, Snijder et al. (2007) found that dairy intake was associated with lower diastolic blood pressure but not with any other parameter of metabolic syndrome while Beydoun et al. (2008) found a protective effect of yogurt. A more recent study evaluating the effects of dairy consumption on metabolic syndrome in more than 7000 middle-aged Koreans concluded that daily intake of dairy products protected against MetS mainly by an association with decreased central obesity (Shin et al., 2013). In summary, the majority of observational data suggest dairy consumption may protect against the development of MetS. There are also some studies that have specifically investigated the effects of yogurt and the incidence of metabolic syndrome. Babio et al. (2015) evaluated the associations between different types of dairy products in a population from the PREDIMED trial. Their results indicate that high consumption of yogurt, whether low or high fat, was correlated with reduced risk of MetS in these individuals characterized by a high risk for heart disease. In contrast, no significant association was found between yogurt consumption and metabolic syndrome in the 6-year SUN cohort follow-up where 306 cases of MetS were identified (Sayón-Orea et al., 2015). Authors used a 136-item validated food frequency questionnaire and logistic models and reported that only one component of MetS, central adiposity, was found to be inversely related to yogurt consumption. However, when yogurt was combined with fruit, an inverse association with MetS was observed (Sayón-Orea et al., 2015).

27.2.2 CARDIOVASCULAR DISEASE

Several reports on diet and cardiovascular health in population studies have established a protective role of yogurt against ischemic heart disease, hypertension, and other cardiovascular problems. For example, a validated food frequency questionnaire was used in 1352 participants from the Observation of Cardiovascular Risk Factors in the Luxembourg survey to determine whether dairy consumption was associated with cardiovascular health (Crichton and Alkerwi, 2014). In this study, cardiovascular health was assessed by seven factors: smoking, body mass index (BMI), physical activity, diet, total cholesterol, blood pressure, and fasting plasma glucose. After controlling for demographics and dietary
variables, those individuals consuming more than five servings per week of dairy products (including yogurt) were found to have better cardiovascular health scores (Crichton and Alkerwi, 2014). Dietary habits of patients with ischemic stroke (n = 300) were analyzed and compared to controls (n = 300) who only differed from stroke patients in having been diagnosed with ischemic heart disease (Rodriguez-Campello et al., 2014). One of the differences reported between patients and controls was that control subjects consumed more probiotic yogurt (Rodriguez-Campello et al., 2014). A recent study on Pakistani urban adults, a population at high risk for hypertension (Mittal and Singh, 2010), studied the relationship between diet and hypertension (Safdar et al., 2015). After analyzing 4304 participants aged 15 years or older and using a 33-item food frequency questionnaire, Safdar et al. (2015) demonstrated that a dietary pattern high in seafood and yogurt was less likely to be associated with hypertension. Similarly, 1390 participants from the multiethnic study of atherosclerosis were given a food frequency questionnaire and correlated specific food intake with measurements of pericardial and hepatic fat (Shah et al., 2015). Results indicated that subjects who consumed greater amounts of fruits, vegetables, and yogurt had less regional adiposity suggesting that these foods can protect against cardiovascular risk as well as diabetes (Shah et al., 2015). In the majority of the analyzed studies, for subjects who were at high risk for heart disease (i.e., hypertension or metabolic syndrome) and those that had documented heart disease, yogurt was highlighted as a good dietary option for cardiovascular health.

27.2.3 TYPE 2 DIABETES

Diabetic patients are characterized by having insulin resistance, dyslipidemias, increased oxidative stress, and low-grade inflammation as well as increased body weight (Ng, 2013). There is substantial information from clinical trials that lifestyle interventions including diet are effective for primary prevention of type 2 diabetes. However, there is high uncertainty regarding specific dietary factors and diabetes prevention. In the case of diabetic patients, yogurt can be beneficial due to its postulated role in gut health, protection of intestinal barrier, and reduction of inflammation (Pei et al., 2015) as well as its proposed healthy effects in obese patients (Pei et al., 2015). There is accumulating evidence that dairy product intake is associated with decreased risk for diabetes (Elwood et al., 2007; Tong et al., 2011). Data derived from the European Prospective Investigation into Cancer (EPIC) have shown that consumption of certain types of dairy products including yogurt may be more relevant for the protection against diabetes (Forouhi, 2015). The EPIC investigators have come to this conclusion after carrying out a very detailed 7-day food dairy record across eight European countries (Forouhi, 2015). Adolescents (12.5–17.5 years old) from the HELENA study, which involves eight European cities, were examined to determine the relationship between dairy consumption and cardiovascular disease and diabetes risk factors (Moreno et al., 2015). Among the biomarkers that were assessed were: body composition, blood pressure, insulin resistance, plasma lipids, and cardiorespiratory fitness in a subset of 511 subjects. Higher consumption of yogurt and of milk and yogurt-based beverages was associated with lower body fat, lower risk for cardiovascular disease, and better fitness (Moreno et al., 2015). A recent study examined whether yogurt consumption was associated with a better diet quality and metabolic profile (risk factors for diabetes) in 6526 participants from the Framingham cohort (Wang et al., 2013). Yogurt consumption was associated with lower concentrations of circulating triglycerides and glucose as well as lower systolic blood pressure and insulin resistance. Results from these epidemiological studies consistently found protective effects of yogurt with the parameters that define diabetes.
CHAPTER 27 YOGURT AND HEALTH BENEFITS

27.3 CLINICAL TRIALS
27.3.1 METABOLIC SYNDROME

Research supports that consumption of dairy products can attenuate many of the clinical biomarkers, which define MetS. Some randomized clinical trials (RCT) specifically have investigated the effect of yogurt intake in subjects having MetS, as defined by the National Cholesterol Education Program Adult Panel III (NCEP ATP III). However, dairy consumption benefits are also shown in RCT with overweight and obese individuals. Other studies also consider the effects of yogurt/dairy products under weight-stable conditions, as weight reduction alone improves MetS components. The literature demonstrates that increased dairy consumption, frequently consumed as a combination of yogurt, milk, or cheese, can attenuate the clinical biomarkers of metabolic syndrome. For example, yogurt or increased dairy consumption of mixed dairy (≥3 servings, compared to ≤1 serving/dairy/day) reduced body weight and fat mass (Zemel et al., 2005), hypertension (van Meijl and Mensink, 2010; Stancliffe et al., 2011; Zemel et al., 2005), and waist circumference (Wennersberg, 2009; Zemel et al., 2005) in weight stable, overweight, or obese adults.

Yogurt’s effect on body mass was studied in 34 obese adults (18–50 year) over a 12-week period. Volunteers consumed a calorie-deficient diet (~500 kcal/day) and were randomized to either a control group taking ~500 mg of a calcium supplement or yogurt, which provided 1100 mg calcium/day (Zemel et al., 2005). The calorie-deficient diet provided ~35% of fat, ~49% carbohydrates, and 16% protein in addition to 8–12 g of fiber/day; macronutrients were maintained constant regardless of study group. The yogurt group consumed three 6-ounce servings of commercial fat-free yogurt daily while the control consumed a placebo of three gelatin servings daily. Body weight, body fat, and fat distribution were measured by dual-energy X-ray absorptiometry, and blood pressure, and serum lipids were measured at baseline and at 12 weeks. Serum lipids and systolic blood pressure remained unchanged, but yogurt consumption significantly lowered diastolic blood pressure (−4.27 mmHg, P < .01). All adults lost weight and body fat due to energy restriction. Yet weight and fat losses were significantly increased by yogurt consumption. Yogurt consumption increased fat loss compared to the control group (−4.43 ± 0.47 vs. −2.75 ± 0.73 kg, P < .005). Trunk fat loss was increased by 81% on yogurt versus control diet (P < .001), and thus there was a greater reduction in waist circumference (−3.00 ± 0.48 vs. −0.58 ± 1.04 cm P < .001). Additionally, lean tissue loss was reduced by 31% on the yogurt diet. Thus, yogurt consumption facilitated fat loss while protecting lean muscle during energy restriction.

The dietary effect of yogurt and milk, versus carbohydrate control foods, on the metabolic parameters including blood pressure, serum lipids, glucose, and insulin was studied in 35 overweight (BMI > 27 kg/m²) males and females (van Meijl and Mensink, 2011). Overweight or obese adults were randomized to consume low-fat milk (2 cups) and yogurt (2/3 cup) or control foods (600 mL juice and 43 g fruit biscuit) for 8 weeks. Following a 2-week washout period, volunteers consumed the alternate diet. Although systolic blood pressure was unchanged, dairy consumption decreased diastolic blood pressure (~2.9 mmHg, P < .027) compared to the control diet. No changes were found in low-density lipoprotein (LDL), but high-density lipoprotein (HDL) was significantly affected (0.04 mmol/L P < .021), which was lower on dairy versus control (0.024; −5.5 to −0.3 mmHg; P < .027). Other parameters including triglycerides and glucose insulin were unchanged.

The effect of increased dairy consumption in diets of those typically consuming a low-dairy diet has also been studied (Crichton et al., 2012; Dugan et al., 2014). In a year-long crossover study
(Crichton et al., 2012), 36 overweight or obese adults (BMI > 25 kg/m²), 18–71 years old, were randomly assigned to consume diets either high or low in dairy foods for 6 months. Following this period of time, they were crossed over to the alternate diet for an additional 6 months. Initial dairy intake of milk, yogurt, or ice cream was < 2 servings/day for all participants. The high-dairy diet included four daily servings of reduced fat milk (250 mL; 1 cup), yogurt (175–200 g), and custard (3/4 cup); and additional dairy intake was limited. On the low-dairy diet volunteers consumed one serving of reduced-fat dairy per day and were instructed to consume a normal diet while limiting dairy to not more than one serving/day. All adults were instructed to continue with normal physical activity throughout the study and energy intake was not restricted. Assessment of waist circumference, body weight, BMI, hip circumference, total body fat, and abdominal fat was conducted at baseline and at 6 and 12 months. Most of the cardiometabolic measures tested were unaltered. There were no differences in baseline values for those assigned high or low dairy. There were also no significant changes in blood pressure, fasting glucose, or serum lipids. Additionally, all anthropometric measurements were not different when consuming high or low dairy. However, the mean change in body weight, BMI, and hip circumference was higher in the high dairy compared to the low dairy. The researchers concluded that these results were due to a small increase in energy intake during the high-dairy phase. Surprisingly, their energy intake difference was reported to be an amount that would cause weight gain of ~ 1 kg/month and so should have resulted in 6 kg difference over the 6-month time frame on the high-dairy diet. Yet the documented weight increase of 1.8 ± 0.4 kg was much smaller than what the increased caloric intake predicted. Since their body weight and percent fat did not change, the researchers attributed the limited weight increase to the effects of calcium in the decrease of fatty acid absorption, which has been shown by others (Denke et al., 1993).

Other researchers aimed to determine the effect of increased dairy intake in typically low-dairy consumers who were classified at having metabolic syndrome (Dugan et al., 2014). Thirty-seven male and female adults, meeting the clinical criteria of MetS (NCEP ATP III), were randomized to incorporate low-fat dairy products (10 oz 1% milk, 6 oz nonfat yogurt, and 4 oz 2% cheese) or control foods (1.5 oz granola bar and 12 oz juice) into their usual diet for 6 weeks. Following a 4-week washout period, subjects consumed the alternate treatment foods for an additional 6 weeks. Subjects received food substitution instruction and were asked to maintain their usual physical activity patterns so that weight was maintained. Anthropometrics, blood pressure, plasma lipids, glucose, and insulin resistance were measured. Metabolic syndrome markers differed by gender with low-fat dairy lowering plasma glucose in men (95.4 ± 9.1 vs. 98.9 ± 10.6 mg/dL, \(P = .048\)) compared to control food intake. In women, low-fat dairy intake lowered waist circumference, BMI, and body weight (\(P < .01\) for all) compared to control food consumption.

The effect of a functional yogurt product verses a conventional yogurt on MetS characteristics in 101 healthy Koreans was tested (Chang et al., 2011). Volunteers aged 20–65 years were randomized to consume two daily servings of either 300 mL of functional yogurt (with additional microorganisms, soluble fiber, an herbal mixture containing yacca and pine needle extract, and whey protein hydroxylate) or a standard yogurt for 8 weeks. All yogurt products were produced by the same manufacture and contained the same macronutrient profile. MetS marker values of body weight, waist circumference, blood pressure, fasting glucose, glycosylated hemoglobin, and lipids were analyzed at baseline (after a 1-week washout) and at week 8. There was no difference between baseline values for adults assigned to receive the different yogurts. However, functional yogurt intake resulted in
decreased body weight ($-0.24 \pm 1.50 \text{ kg}$, $P = .006$) and BMI ($-0.10 \pm 0.58 \text{ kg/m}^2$, $P = .006$) compared to the control group. Although LDL decreased in both groups, the lowering effect was greater in the functional group ($7.71 \pm 14.14 \text{ mg/dL}$; $P < .001$) from baseline when the control group was also compared to baseline ($0.43 \pm 15.32 \text{ mg/dL}$; $P = .84$). This difference in change of LDL between groups was significant ($P = .044$ by independent t-test). These results highlight a synergistic effect between yogurt consumption with additional added food components. Some of these food components, such as fruits, a good source of soluble fiber, may be added to yogurt thus improving the effects caused by yogurt consumption alone.

The question of how much dairy needs to be consumed to observe health benefits and whether these benefits will continue was researched in adults with MetS (Stancliffe et al., 2011). Adults meeting NCEP ATP III MetS classification ($n = 40$) were randomly assigned to consume diets containing either an adequate dairy intake (3.5 daily servings of milk and yogurt) or low-dairy intake (<0.5 servings) with nondairy food substitutions (fruit cups, granola bars) foods with a weight-maintenance diet. Markers of inflammation and oxidative stress were measured during baseline, at day 7 and weeks 1, 4, and 12. The higher dairy intake lowered oxidative markers following 1 week of intake with a 35% decrease in malondialdehyde and 11% in oxidized LDL ($P < .01$). This effect continued with 25% decrease in oxidized LDL by 4 weeks, however, no differences were found in oxidative markers on the low-dairy intake. Similarly, higher dairy intake reduced systemic inflammation following 4 weeks of intake with decreases in tumor necrosis factor (TNF)$\alpha$ (35%, $P < .05$), interleukin-6 (IL-6) (21%; $P < .02$), and monocyte chemoattractant protein-1 (MCP-1) (14% $P < .05$). These benefits also continued at 12 weeks with further decreases in MCP-I (24%, $P < .05$) and circulating C-reactive protein (CRP) (47%, $P < .005$). Additional MetS markers were also positively affected with higher dairy intake. There was no effect on fasting glucose but higher dairy significantly lowered insulin starting at day 7 and continuing throughout the study (lowered $-0.83 \text{ mmol/L}$ day 7, $-0.28$ at day 84; $P < .05$). HOMA-IR also improved (lowered $-0.59$ $P < .05$). Neither diet affected body weight; however, higher dairy reduced waist circumference and trunk fat ($P < .01$ for both).

### 27.3.2 Cardiovascular Health

Yogurt was tested as part of a low-glycemic preload snack food on the effect of various cardiovascular risk factors (Azadbakht et al., 2013). The preload snack consisted of a combination of foods with a low-glycemic index snack (yogurt and vegetable salad) on cardiovascular disease risk factors and anthropometrics. Sixty-five adults (25 men and 35 women) consumed a prepared hypocaloric diet for 3 months and were randomized to consume the preload either 15 min before the meal or to consume the low-glycemic test foods with the meal directly. Meals for both groups contained the same macronutrients. There was a greater reduction of cardiovascular risk factor in those consuming the preload compared to those consuming the low-glycemic foods directly with the meal. Specifically, preload consumption decreased waist circumference ($-7.8 \text{ cm} \pm 0.5\%$), body weight ($-2.7 \text{ kg} \pm 0.2\%$), systolic blood pressure ($-2.7 \text{ mmHg} \pm 0.2\%$), and serum lipids triglycerides and HDL-cholesterol ($-3.1 \text{ mg/dL} \pm 0.53\%$, $-4.4 \text{ mg/dL} \pm 0.4\%$; $P < .05$ for all), compared to consuming them with a meal. Additionally, only those consuming a preload experienced a reduction in fasting glucose and LDL cholesterol. Consumption of yogurt with other low-glycemic foods as a preload snack furthered combined weight loss benefits in reducing heart disease risk factors.
Dairy products contain high amounts of saturated fatty acids thus their intake has been discouraged as a dietary treatment for cardiovascular disease prevention. However, current research indicates that total dairy products are not associated with increased artery thickening (Ivey et al., 2011). Specifically, yogurt consumption has shown to be protective of the carotid artery intima thickness, thus attenuating heart disease risk. Dietary analysis was conducted in 1080 women (70 years) in Australia with a validated food frequency questionnaire (Ivey et al., 2011). Dairy food intake including milk, yogurt, and cheese was determined. Carotid artery intima thickness was examined 3 years later with ultrasound. Serum lipids and blood pressure were evaluated at baseline. No association was found between total dairy consumption and carotid artery intima thickness ($P = .05$). However, yogurt consumption, but not that of other dairy foods, was negatively associated with artery intima thickness (Ivey et al., 2011). Yogurt intake was then evaluated by quantity consumed with intake categorized as low (100 g/day), moderate (100–200 g/day), or high (200 g/day). Those consuming increased yogurt consumption (>100 g yogurt/day) had a significantly lower artery intima thickness than those with lower yogurt consumption (<100 g/day; $P = .002$). This association remained significant after adjusting for dietary and lifestyle factors including age, BMI, type 2 diabetes, never smoking, history of vascular disease, macronutrient intake, and physical activity (Ivey et al., 2011).

### 27.3.3 DIABETES

van Meijl and Mensink (2010) determined the effect of dairy on inflammation in obese (BMI 31.1 men, 32.4 women) subjects (n = 35; aged 18–70). Subjects were randomized to consume dairy foods (500 mL low-fat milk and 150 g low-fat yogurt; ~3 servings dairy/day) or control foods (600 mL fruit juice and 43 g fruit biscuits) in a crossover fashion with 8 weeks of intervention separated by a two-week washout. Body weight was not different at the end of each intervention. Energy content of products was similar (360 kcal dairy vs. 405 kcal control). Dairy consumption significantly ($P = .027$) decreased systolic blood pressure by 2.9 mmHg ($P = .027$) and trended toward decreasing ($P = .09$) diastolic blood pressure by 2.9±5.2 mmHg, but did not alter other components of MetS. Inflammatory markers C-reactive protein, interleukin-6, monocyte chemoattractant protein-1, intercellular adhesion molecule-1, and vascular cell adhesion molecule-1 were not changed. There was a trend for TNFα to be lower following dairy consumption ($P = .070$). It is not clear if the subjects ate dairy other than what was provided to them. In fact, researchers state that “subjects drank more milk during the control periods.” Additionally, comparing the mean daily intake of calcium, excluding calcium from the intervention foods, shows that subjects had greater calcium intakes on the control diet than while on the dairy diet. Thus there may not have been a large enough difference between calcium intakes to capture differences in inflammatory compounds.

Increased insulin secretion is especially important in those with T2DM as they typically experience a decreased insulin response to carbohydrate (CHO). This beneficial effect of combining the insulin-stimulating effects of whey with CHO intake was demonstrated by Manders et al. (2005). These authors provided CHO alone or with a protein hydrolysate/amino acid mixture to 10 volunteers with type 2 diabetes and measured the plasma insulin response from CHO with and without the protein/amino acid mixture. Consuming protein/amino acids with CHO resulted in higher (299 ± 64% vs. 132 ± 63%; $P < .001$) plasma insulin and reduced plasma glucose (28 ± 6% vs. 33 ± 3%), compared to those consuming CHO alone. Further, isotope tracers ([6,6-$^{2}$H$_{2}$] glucose)
used to measure glucose disposal found that diabetics consuming protein with CHO experienced a significant increase (13 ± 3%; \( P < .01 \)) in glucose disposal compared to those consuming CHO alone.

Seventy first-time pregnant women in their third trimester were randomly allocated to consume either regular yogurt or a probiotic-enhanced yogurt (Asemi et al., 2013). All women consumed 200 g of their assigned yogurt for 9 weeks. They continued their usual physical activity and were instructed to avoid all other fermented or probiotic-containing foods. Probiotic-enhanced yogurt contained additional *Lactobacillus acidophilus* and *Bifidobacterium animalis*, providing a total count of at least \( 1 \times 10^7 \) cfu. Fasting plasma glucose and serum insulin was measured at study baseline and at the 9-week end point. Insulin was unaffected by both yogurts, but there was greater change from baseline in serum insulin in those consuming probiotic yogurt (+1.2 ± 1.2) compared to those with conventional yogurt (+5.0 ± 1.1 \( \mu \)IU/mL, \( P = .02 \)). These results were similarly reflected in the change from baseline for the homeostatic assessment–insulin resistance score in probiotic yogurt (−0.2 ± 0.3) and regular yogurt intakes (0.7 ± 0.2, \( P = .01 \)).

The effect of yogurt and probiotic-enhanced yogurt was also examined in diabetic patients. Sixty (30–60 years) adults diagnosed with type 2 diabetes and who did not consume probiotic yogurt or probiotic products were randomized to consume 300 mL regular yogurt or probiotic-enhanced yogurt for 6 weeks (Ejtahed et al., 2012). Probiotic-enhanced yogurt contained added *L. acidophilus* La5 and *Bifidobacterium lactis* Bb12. Volunteers maintained their usual physical activity and avoided other yogurt foods. Compared with those consuming the regular yogurt, those eating probiotic yogurt had decreased fasting glucose (\( P < .01 \)) and glycosylated hemoglobin (\( P < .05 \)). Antioxidant status was also improved in the probiotic yogurt group, with increased activities in glutathione peroxidase and superoxide dismutase activities and total antioxidant status (\( P < .05 \)). In accordance with increased antioxidant capacity serum malondialdehyde also significantly decreased (\( P < .05 \)). Importantly, this decreased oxidant marker was found in both yogurt groups. Thus while probiotic yogurt protects serum glucose and insulin more than regular yogurt, benefit is still seen by increased regular yogurt intake.

Collectively, these data support a beneficial role of yogurt and other dairy products in the attenuation of inflammatory markers, oxidative stress, and glucose control in healthy or overweight and obese individuals with components of MetS and the resultant comorbidities CVD and T2DM. In addition, it has been reported that that obese subjects provided either a hypocaloric diet with yogurt (providing 100 mg calcium/day) or a eucaloric diet with high dairy (1200 mg calcium/day with three servings of dairy) had lower serum CRP (\( P < .05 \)) and higher adiponectin (\( P < .05 \)), regardless of weight loss status (Zemel and Sun, 2008). The main randomized clinical trials with yogurt/dairy and the health outcomes are presented in Table 27.1.

### MECHANISMS OF ACTION BY COMPONENTS OF DAIRY/YOGURT

#### 27.4.1 DAIRY AND CALCIUM EFFECTS ON ADIPOSY

Calcium is a component of yogurt (dairy) that has been postulated to promote weight reduction through the tightly regulated modulation of plasma 25-hydroxyvitamin D concentrations via parathyroid hormone (PTH). PTH increases renal calcium reabsorption and activates bone resorption and the kidney
<table>
<thead>
<tr>
<th>Study</th>
<th>Population</th>
<th>Dairy Type and Intake</th>
<th>Health Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zemel et al. (2005)</td>
<td>RCT—parallel design N = 34—trial 1 N = 29—trial 2</td>
<td>Three dairy servings/day—one milk; low calcium vs. high calcium; trial 2—same dairy but also calorie restriction</td>
<td>HD group decrease in body fat, trunk fat, insulin, BP; lean mass was increased; there were no changes in low-dairy group</td>
</tr>
<tr>
<td>van Meijl and Mensink (2010)</td>
<td>RCT-crossover study N = 35 overweight or obese, male/female</td>
<td>Low-fat milk (2 cup) and low-fat yogurt (2/3 cup) or fruit juice (600 mL) and fruit biscuit for 8 weeks</td>
<td>Dairy consumption decreased systolic blood pressure by 2.9 mmHg No change in other MetS markers</td>
</tr>
<tr>
<td>Crichton et al. (2012)</td>
<td>RCT-crossover study N = 36 overweight or obese adults</td>
<td>4 serv./day reduced fat dairy vs. low dairy ≤1 serv./day</td>
<td>No difference in WC, BW, fat mass Study did not restrict energy intake Dairy decreased systolic BP</td>
</tr>
<tr>
<td>Dugan et al. (2014)</td>
<td>RCT-crossover study N = 37 male/female</td>
<td>3 serv./day: 1% milk, 6 oz nonfat yogurt, 4 oz 2% cheese; or control: 1.5 oz granola bar and 12 oz juice</td>
<td>Low-fat dairy decreased plasma glucose in men and WC, BMI, and BW in women</td>
</tr>
<tr>
<td>Chang et al. (2011)</td>
<td>RCT-parallel design N = 35 healthy adults</td>
<td>Consumed functional yogurt (300 mL) or placebo yogurt 2×/day</td>
<td>Functional yogurt reduced body weight, BMI, and LDL cholesterol</td>
</tr>
<tr>
<td>Stancliffe et al. (2011)</td>
<td>RCT, parallel N = 40 with MetS</td>
<td>Weight maintenance diets Low dairy (&lt;0.5 serv./day and &lt;600 mg Ca) Adequate-dairy (&gt;3.5 serv./day and ≥600 mg calcium)</td>
<td>AD—decreased MNDA and oxidized LDL; decreased TNFα, IL-6, MCP1; reduced wc and trunk fat, reduced plasma insulin and insulin sensitivity LD—no change</td>
</tr>
<tr>
<td>Zemel and Sun (2008)</td>
<td>Parallel design obese adults</td>
<td>Three dairy servings/day—one milk Milk, yogurt, and cheese</td>
<td>Dairy and hypocaloric diet decreased CRP</td>
</tr>
<tr>
<td>van Meijl and Mensink (2010)</td>
<td>RCT N = 35, overweight adults</td>
<td>Low-fat milk and low-fat yogurt or fruit juice and fruit biscuit for 8 weeks</td>
<td>Decreased TNFα No effect on CRP, PAI-1, IL-6, MCP-1, or VCAM-1</td>
</tr>
<tr>
<td>Manders et al. (2005)</td>
<td>RCT N = 10 adults with T2DM</td>
<td>Provided CHO alone or with a protein hydrolysate/amo acid mixture</td>
<td>Consuming protein/amino acids with CHO resulted in higher plasma insulin Reduced plasma glucose (28±6% vs. 33±3%), vs. consuming CHO alone</td>
</tr>
<tr>
<td>Asemi et al. (2013)</td>
<td>RCT N = 70 third trimester pregnant women</td>
<td>Provided 200 g of probiotic-enhanced yogurt or regular yogurt</td>
<td>There was no change in serum insulin, HOMA at 6-week vs. baseline following enhanced yogurt Serum insulin levels, HOMA were lower following PB yogurt vs. regular yogurt</td>
</tr>
<tr>
<td>Ejtaheh et al. (2012)</td>
<td>RCT—parallel design N = 64 adults with T2DM</td>
<td>Intervention: 300 g/day of probiotic yogurt Control: 300 g/day of conventional yogurt for 6 weeks</td>
<td>Fasting glucose, HbA1C were decreased and erythrocyte SOD, glutathione peroxidase, and total antioxidant activity were increased following probiotic yogurt compared to control</td>
</tr>
<tr>
<td>Azadbakht et al. (2013)</td>
<td>RCT N = 65 adults</td>
<td>Preload with yogurt, and vegetable salad with hypocaloric diet</td>
<td>BW, WC, TG, TC, and SBP were lower in preload group vs. control Fasting glucose and LDL-cholesterol decreased only in the preload group</td>
</tr>
</tbody>
</table>

AD, adequate dairy; BP, blood pressure; BW, body weight; CHO, carbohydrate; CRP, C-reactive protein; HbA1C, glycosylated hemoglobin; HD, high dairy; HOMA, homeostatic model assessment; IL-6, interleukin 6; LD, low dairy; MCP-1, monocyte chemoattractant protein-1; MetS, metabolic syndrome; RCT, randomized clinical trial; SBP, systolic blood pressure; SOD, superoxide dismutase; T2DM, type 2 diabetes mellitus; TC, total cholesterol; TG, triglycerides; WC, waist circumference.
hydroxylase enzyme, which converts inactive vitamin D into its active form. Thus, lower plasma calcium triggers an increase in the synthesis of 1, 25-OH₂-vitD, which causes an influx of calcium ions into adipocytes resulting in increased intracellular calcium. Intracellular calcium activates gene transcription of fatty acid synthase (FAS) within the adipocyte and inhibits lipolysis through activation of phosphodiesterases and inhibition of hormone-sensitive lipase (Zemel, 2003). Thus a theory has been proposed that when dietary calcium is increased, intracellular calcium is lowered therefore there is mitigation of adiposity via decreases of de novo lipogenesis and direct stimulation of lipolysis (Zemel, 2003).

This proposed mechanism was demonstrated in a transgenic mouse model for diet-induced obesity (Shi et al., 2001). Mice were fed diets containing either low calcium, supplemental calcium, medium dairy, or high dairy for 6 weeks. Fat pads were harvested to quantify fat mass and to determine fatty acid synthase expression and activity levels in adipocytes. Mice fed low-calcium diets had higher body weight gain and fat mass compared to those fed supplemental calcium or dairy. Both supplemental calcium and high dairy decreased FAS activity by 35% and 65%, respectively (Shi et al., 2001).

Treatment of human adipocytes with 1, 25-OH₂-vitD has also resulted in increases in fatty acid synthase and inhibition of lipolysis (Shi et al., 2002). However, this theory has not been fully supported by human studies (Shockravi et al., 2008; Dugan et al., 2014). Furthermore, the previous mechanism is incongruent with epidemiological data that demonstrate lower levels of vitamin D in overweight and obese populations (Parikh et al., 2004; Ganji et al., 2011).

27.4.2 DAIRY INTAKE AND BLOOD PRESSURE

Different mechanisms have been suggested to account for the blood pressure–lowering effect of dairy products. The most accepted mechanism involves inhibition of the angiotensin-converting enzyme (ACE), which plays a major role in the activation of angiotensin (Ma et al., 2010). Several proteins in food have been identified as ACE inhibitors including those from dairy (Majumder and Wu, 2014). Peptide sequences believed to be responsible for ACE inhibition include the lactotripeptide amino acid sequences isoleucine-proline-proline and valine-proline-proline (Phelan and Kerins, 2011). Data from clinical interventions support a blood pressure–lowering effect of dairy, specifically from low-fat dairy and from the whey fraction (Pal and Ellis, 2010).

27.4.3 EFFECT OF DAIRY INTAKE ON SERUM GLUCOSE LEVELS

The high–amino acid content, specifically the branch chain amino acids in dairy, may modulate glucose levels by increasing postprandial insulin secretion. Increased dairy intake has been shown to lower serum glucose through several potential mechanisms, which may include protein-induced increases in serum insulin and increasing hepatic regulatory control of glucose production.

Whey protein, for example, has been shown to increase glucose-dependent insulinotropic polypeptide, which triggers insulin release by pancreatic β cells (Graf et al., 2011), a mechanism that might be beneficial for diabetic patients. For example, Frid et al. (2005) demonstrated that whey increased insulin and decreased postprandial response in diabetic patients when provided at breakfast in combination with high-glycemic foods. Further, when isotope tracers were used to measure glucose disposal
[(6,6-^2^H_2^) glucose], consumption of protein with carbohydrate resulted in an increase \((P < .01)\) in glucose disposal compared to intake of carbohydrate alone (Manders et al., 2005).

Layman et al. (2003) have also postulated that when carbohydrates are replaced with protein, the branched-chain amino acids, specifically leucine, will “shift” the regulatory control of glucose homeostatic away from the pancreas and insulin secretion toward hepatic control through gluconeogenesis. After feeding a 400 kcal breakfast with either high protein or high carbohydrate to 24 women for breakfast, insulin increased more than 2-fold and was >40% higher in the high-carbohydrate group and plasma branched-chain amino acids were lower compared to the high-protein group (Layman et al., 2003). Leucine has also been shown to exert a protective effect against insulin resistance and weight gain in mice fed a high-fat diet (Macotela et al., 2011), although more studies on the specific effects of leucine are still needed.

27.4.4 CALCIUM, DAIRY AND PLASMA LIPIDS, LIPOPROTEINS, AND APOLIPOPROTEINS

High-calcium content and the lipid fractions present in dairy products may positively alter the serum lipid profile. The postulated mechanisms are intestinal binding of calcium to saturated fatty acids, which forms insoluble soaps that are excreted in the feces or calcium binding to bile acids, which leads to the interruption of the enterohepatic circulation and the removal of circulating cholesterol via upregulation of hepatic LDL receptors (Abedini et al., 2015).

In a randomized trial, healthy men with moderately high plasma cholesterol received either high or low calcium (Denke et al., 1993). Higher calcium intake resulted in greater excretion of palmitic, stearic, and oleic acids as well as lower total and LDL cholesterol and apolipoprotein B (Denke et al., 1993). Results from a meta-analysis aimed at determining the effect of calcium on fetal fat excretion concluded that an increase in 1240 mg of dairy calcium per day corresponds to 5.2 g/day increase in fat in the feces (Christensen et al., 2009). These cholesterol-lowering effects of calcium may attenuate the potential cholesterol-raising effects of palmitic, myristic, and lauric acids found in dairy foods. Specific fatty acids provided by dairy may also modulate lipoprotein metabolism. Other lipid components in milk including phospholipids and sphingolipids have been reported to decrease cholesterol absorption in animals (Kamili et al., 2010).

27.5 CONCLUSION

It is clear from all the evidence derived from studies described in this chapter that dairy and yogurt have protective effects against MetS by decreasing blood pressure (Shockravi et al., 2008) and fasting glucose (Elwood et al., 2007) and by helping in the maintenance of a healthy body weight (Snijder et al., 2007; Shin et al., 2013). In terms of beneficial effects on cardiovascular health, it has been shown that yogurt may decrease inflammation (Dugan et al., 2016), oxidative stress (Ejtahed et al., 2012), and improve dyslipidemias (Ganji et al., 2011). Finally, yogurt has been shown to decrease insulin resistance (Sayón-Orea et al., 2015; Wang et al., 2013) and glycosylated hemoglobin (Mohamadshahi et al., 2014) in patients diagnosed with diabetes. A summary of the effects of yogurt in all these conditions is depicted in Fig. 27.1.
CHAPTER 27 YOGURT AND HEALTH BENEFITS

REFERENCES


FIGURE 27.1

Yogurt has been found to have effects on: Cardiovascular disease by decreasing inflammation, oxidative stress, dyslipidemias, and blood pressure; on Diabetes by lowering insulin resistance, glycosylated hemoglobin, and plasma glucose; on Obesity by decreasing central fat and body weight; and on Metabolic Syndrome by decreasing waist circumference (WC), blood pressure, plasma triglycerides, and fasting glucose.


Kamili, A., et al., 2010. Hepatic accumulation of intestinal cholesterol is decreased and fecal cholesterol excretion is increased in mice fed a high-fat diet supplemented with milk phospholipids. Nutr. Metab. 7 (1), 90.


Pal, S., Ellis, V., 2010. The chronic effects of whey proteins on blood pressure, vascular function, and inflammatory markers in overweight individuals. Obesity (Silver Spring Md.) 18 (7), 1354–1359.


ROLE OF YOGURT IN THE NUTRITION AND HEALTH OF CHILDREN AND ADOLESCENTS

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28.1 INTRODUCTION

Yogurt is a nutrient-rich food containing a combination of nutrients that are important for growth and development during childhood and adolescence. Proper nutrition at these key times promotes health in adulthood. While undernutrition is still a prominent concern in developing countries leading to stunting, emaciation, and wasting (Administrative Committee on Coordination Sub-Committee on Nutrition, 2000), overconsumption of energy-dense and nutrient-poor foods, beverages, and snacks has exacerbated the prevalence of childhood obesity in developed nations. Stabilization of childhood obesity prevalence in some countries such as the United States has shifted the focus from merely attaining energy balance to achieving healthy eating and physical activity patterns with the goal of not only optimizing growth and development, but also minimizing the risk of developing chronic diseases (Ogata and Hayes, 2014). A whole-diet approach to healthy eating, which includes dairy products such as yogurt, can help meet nutrient needs, particularly shortfall nutrients such as vitamin D, vitamin E, magnesium, calcium, vitamin A, vitamin C, folate, fiber and potassium, and iron for adolescent females, without exceeding energy requirements. Shortfall nutrients such as potassium, calcium, and vitamin D as well as fiber are of particular public health concern because of their association with various adverse health outcomes (Freeland-Graves and Nitzke, 2013; U.S. Department of Health and Human Services and U.S. Department of Agriculture, 2015). Furthermore, while dairy products are part of a healthy eating pattern, they have also been identified as a food group that is consumed in suboptimal quantities to amounts recommended by many national food-based dietary guidelines. The present text will discuss (1) the nutritional value of yogurt, (2) its contribution to shortfall nutrients and recommended intakes of dairy products, (3) its role within healthy eating patterns, and (4) its association to diet-related health issues in children and adolescent populations.

28.2 NUTRITIONAL VALUE OF YOGURT

As a source of multiple macronutrients and micronutrients, yogurt is a nutrient-rich food for children and adolescents without being an excessive source of calories or fat. The combination of high quantities of desirable nutrients and relatively low amounts of fat and sugar qualify yogurt as a nutrient-dense food, particularly unsweetened and low-fat yogurts (Nicklas et al., 2014). The contribution of 100 g of yogurt to the energy and macronutrient needs of children 4–6 and 7–11 years is illustrated in Fig. 28.1A and B.
**FIGURE 28.1**
Percent contribution of 100g of commonly consumed yogurts to the reference nutrient intakes for energy, fat, carbohydrates, and protein in (A) children (4–6 years) and (B) adolescents (7–11 years). *The reference nutrient intake for fat is based on the upper range of recommended intake (35% of calories consumed). **The reference nutrient intake for protein is based on minimum requirements for growth, development, and lean muscle maintenance (g/kg of weight depending on age).

From these figures, it can be seen that yogurt can be an excellent source of protein, and even whole milk yogurts do not appear to contribute to excessive caloric or fat intakes; however, the impact of added sugar and saturated fat intakes from yogurts to children’s diets is not entirely clear. In one study, yogurts (including fromage frais and dairy desserts) were a significant source of nonintrinsic sugars for very young children in the United Kingdom, but this was not the case for older children or adolescents. This may reflect, however, the low-diet diversity at this young age and higher level of intake of yogurt among younger children (Williams et al., 2015).

Fig. 28.2 illustrates the contribution of 100 g of low-fat fruit yogurt to the vitamin and mineral intakes of children and adolescents. Yogurt is an excellent source of iodine, vitamin B12, phosphorus, calcium, riboflavin, and thiamin for children and adolescents as well as a source of folate, magnesium, potassium, and selenium. Yogurt provides negligible amounts of iron, niacin, and vitamin B6. Generally, for younger children in particular, yogurt is a concentrated source of nutrients providing a higher percentage of reference nutrient intakes proportional to children’s

![Figure 28.2](image)

**FIGURE 28.2**
Percent contribution of 100 g of low-fat fruit yogurt to the mineral and vitamin requirements of children and adolescents 4–6, 7–10, 11–14, and 15–18 years. Reference values for each age group are average values of both sexes and each year in the age group.

requirements. In countries where milk products are fortified with vitamins, yogurt is a particularly interesting vehicle for vitamin D fortification and has been highlighted as such by the Dietary Guidelines Advisory Committee (U.S. Department of Health and Human Services and U.S. Department of Agriculture, 2015).

Unsweetened yogurt provides a source of simple carbohydrates, primarily in the form of lactose, which is known to enhance the absorption of calcium and magnesium. Lactose is slightly less calorific than other sugars and has a lower glycemic index (46) compared to glucose (100) and sucrose (60) (Chandan and Kilara, 2011), which may contribute to satiety, particularly in combination with whey proteins found in yogurt (Dougkas et al., 2011). Lactose intolerance is commonly cited as a barrier to milk consumption, and children who follow dairy-free diets are known to have lower bone mineral density (Black et al., 2002; Du et al., 2002; Rockell et al., 2005). Yogurt containing standard starter cultures, Lactobacillus delbrueckii ssp. bulgaricus and Streptococcus thermophilus, improve digestion of lactose for individuals with lactose intolerance (EFSA Panel on Dietetic Products, 2010). Because of these physiological benefits, yogurt, containing standard starter cultures, may be classified as a probiotic for lactose maldigesters (Hill et al., 2014). Consuming yogurt instead of milk can relieve or eliminate gastrointestinal symptoms associated with lactose intolerance, allowing children to take advantage of the nutritional benefits of dairy food intake, which include calcium, protein, vitamins, and minerals (Chandan and Kilara, 2011).

While unsweetened low-fat yogurt can universally be considered as a nutrient-dense food, it is less clear whether sweetened or whole-fat yogurts can be classified with the same auspicious label given that the addition of caloric sweeteners and the higher content of saturated fat generally diminish the nutrient density profile of foods (Darmon et al., 2009; Fulgoni et al., 2009). Nevertheless, there is little indication that children benefit from strict fat guidelines. Furthermore, full-fat dairy foods such as whole-milk yogurt contain bioactive fats that may be beneficial for certain populations (Drehmer et al., 2016). The World Health Organization (WHO) has recommended strict limitations to intakes of added sugars for children, which should account for less than 10% of dietary energy. This recommendation is largely based on evidence indicating a positive association between sugar-sweetened beverage intake and overweight or obesity in children (WHO, 2015). However, little is known about the effects of sweetened dairy products on health, and at this time it is unclear whether the same blanket warnings on products with added sugar should systematically be applied to nutrient-rich products such as yogurt. There is a belief that such restrictions are not feasible for the average person and may discourage the public from consuming nutrient-rich foods (Erickson and Slavin, 2015).

Yogurt protein, like milk protein, is of high quality, providing all essential amino acids, which optimally support the growth of children and adolescents. This high quality is of added value, particularly in low-income and developing countries, to protect against malnutrition as a result of protein-deficient diets (Muehlhoff et al., 2013). Milk protein contains a combination of whey (20%) and casein (80%), which plays a role in optimal calcium and phosphate absorption (Haug et al., 2007), provides precursors for bioactive peptides that are released during yogurt fermentation, and affords potential health benefits on the immune and digestive systems (Nagpal et al., 2011). The high whey content of yogurt may help to regulate food intake by activating satiety signals. Furthermore, whey contains a high concentration of branched chain amino acids, particularly leucine, which are involved in lean body mass preservation and promoting protein synthesis (Marette and Picard-Deland, 2014). In developed countries where the protein quality of the diet is not of concern, there have been recommendations against very high protein intakes in early childhood (e.g., from cow’s milk); grade 1 evidence is convincing, showing that higher protein intakes in infancy and early childhood are linked to increased growth
and/or body mass index (BMI) in childhood. However, there does not appear to be any evidence that high-protein intakes are related to adiposity at any age or BMI in later childhood (Hörnell et al., 2013).

### 28.3 YOGURT AND DAIRY CONSUMPTION

Most data on yogurt, milk, and dairy consumption in children have involved American cohorts; however, studies from other countries will be described when available. Over the last several decades, the intake of dairy products has generally declined in young populations from industrialized countries. Dairy product intake further decreases with increasing age, attributed in part to increased soda consumption during adolescence and less frequent breakfast consumption. Food-based dietary guidelines vary slightly by country but generally recommend that children <9 years consume 2–3 servings and adolescents consume 3–5 servings of dairy products (Dror and Allen, 2014). Dietary guidance for the consumption of dairy products is largely based on bone health and the importance of accrual of optimal bone mass during youth. Close to 50% of adult peak bone mass is accrued during teenage years, making adequate dairy intake important during childhood and adolescence. There is also evidence from developing countries of better growth in children who consumed dairy products (Weaver, 2014).

In a cross-section of Americans, it was found that milk, cheese, and yogurt accounted for 55% of dietary calcium intake for children (Cluskey et al., 2015). In Japan, where dairy products are less consumed, dairy products account for 30% of dietary calcium (Uenishi and Nakamura, 2010). Many children and adolescents do not consume adequate amounts of dairy products, which potentially results in compromised nutritional status from decreased intakes of important vitamins and minerals, particularly calcium. According to the Scientific Report of the 2015 Dietary Advisory Committee, “increasing low-fat/fat-free fluid milk and yogurt and decreasing cheese would result in higher intakes of magnesium, potassium, vitamin A and vitamin D while simultaneously decreasing intake of sodium and saturated fat” (U.S. Department of Health and Human Services and U.S. Department of Agriculture, 2015). Yogurt consumption specifically can contribute to increased intakes of key shortfall nutrients for children such as calcium, potassium, folate, and vitamin B12. In a population of children and adolescents from northeastern England, yogurt was consumed by 56% of participants (Clerfeuille et al., 2013; Green et al., 2015). Consumption of yogurt in other countries may be much lower because of lack of availability, popularity, or cultural perceptions about the product. For example, yogurt consumption is very low in the United States and does not contribute in any considerable manner to intakes of dairy products in the American population at any age group (Quann et al., 2015), but encouraging consumption of one cup a day can help children meet nutrient requirements to improve diet quality (Webb et al., 2014). In Brazil, 80% of the population do not meet recommendations for dairy product intake (Verly Junior et al., 2013). Dairy products in general are only consumed by 43% of the population in Brazil. Milk and cheese are the primary dairy products consumed, and yogurt is only consumed by approximately 7% of Brazilians (Possa et al., 2017). Mostly only ultraprocessed yogurts and specialty diet yogurts are available in Brazil, and they are not considered as a breakfast food or dessert given to children. In fact, there are food-safety concerns over keeping yogurt at school without proper refrigeration. While plain unsweetened yogurt is recommended in the dairy food category of Brazilian food guidelines, it is not a product that is readily available or might just be available to higher social classes. Only recently have pediatricians in Brazil been recommending yogurt as part of the arsenal for calcium intake, but unsweetened unflavored yogurt is emphasized (Fisberg, personal communication).
In an American study, yogurt consumption in children (8–18 years) was associated with higher intakes of calcium, vitamin D, and protein and lower intakes of saturated and total fat, demonstrating yogurt’s potential to contribute to intakes of shortfall nutrients, particularly those involved in bone health (Keast et al., 2015). A Chinese study with preschool children (3–5 years) demonstrated that supplementing the usual diet with a 125 g serving of yogurt daily resulted in higher intakes of calcium, zinc, and vitamin B12 than in the control group. Furthermore, bone mineral density after 9 months of supplementation was significantly greater in the yogurt-supplemented group (He et al., 2005). In Japan, yogurt and milk consumption were independently associated with measures of bone strength in a representative group of adolescents (Uenishi and Nakamura, 2010). Furthermore, yogurt consumption has been linked to dental health: reduced tooth erosion and fewer dental carries (Dror and Allen, 2014; Salas et al., 2015).

28.4 HEALTHY DIETARY BEHAVIORS

Little is known about how and when yogurt is consumed by young populations. There are some studies that model yogurt as a snack food, a dessert, or a milk alternative at breakfast. Among adults, yogurt appears to be a signature of healthy lifestyles and behaviors (Panahi et al., 2016) because it is often associated with healthy dietary patterns, more physical activity, less smoking, higher fruit and vegetable intake, more education, and higher income (Wang et al., 2013; Martinez-Gonzalez et al., 2014; D’Addezio et al., 2015; Possa et al., 2015; Ricceri et al., 2015). Possa et al. (2015) observed that yogurt consumers had a higher educational level (≥8 years: 83.8% vs. 79.9%), a higher frequency of individuals working and/or currently studying (67.7% vs. 65.5%), were more physically active at leisure time (17.2% vs. 14.3%), had reduced alcohol intake (3.6 g/day vs. 6.4 g/day), and had a lower frequency of smoking (21.7% vs. 25.5%) compared to nonconsumers (P < .05). Few studies have investigated younger populations, but there is a good indication that yogurt consumption in children and adolescents may mirror what is seen in adult populations. For example, in one American study, children who consumed yogurt as infrequently as once per week had higher diet quality scores than nonconsumers. Yogurt consumers were also more likely to meet physical activity requirements, be non-Hispanic Whites, and have higher income-to-poverty ratios (Zhu et al., 2015).

In the United States, many children skip breakfast. This behavior has been linked to lower intakes of many nutrients, poorer diet quality, and increased prevalence of being overweight and obese. The promotion of breakfast to children and adolescents and the inclusion of yogurt as a milk alternative can help improve nutritional status and promote healthy weights in these young populations (Webb et al., 2014). Among European adolescents, ready-to-eat cereal consumption was associated with higher milk and yogurt intakes and better diet quality (Michels et al., 2015a,b).

Snacking is common in Western countries and snacking frequency as well as the number of individuals who snack has increased significantly over the last several decades and can represent up to 25% of the daily caloric intake of children and adolescents (Jahns et al., 2001). Snacking, per se, is not harmful, particularly when healthy snacks contributing to key nutrient intakes are selected; however, trends toward frequent snacking of nutrient-poor energy-dense snacks has led to increased intakes of calories and added sugars (Larson and Story, 2013). Replacing these snacks with nutrient-dense foods can help reduce excess caloric and added sugar intake as well as increase intakes of nutrients that are deficient in the diet. Low-fat, plain, or even sweetened yogurt is a reliable alternative to nutrient-poor, energy-dense snacks for children (Hess and Slavin, 2014). Regular or whole-fat yogurt, however, is considered an energy-dense snack (Longley et al.,
It should be noted that flavored sweetened yogurts contain a wide variation of added sugar (Erickson and Slavin, 2015) and may not be appropriate as a snack for children, especially young children. In Brazil, food-based dietary guidelines specify that sweetened and flavored yogurts are classified as ultraprocessed foods, and as such should be limited or avoided (Ministry of Health of Brazil, 2014). In adult populations, associations between snacking and adiposity appear to be driven by BMI status and snack quality (O’Connor et al., 2015). These associations are likely to be similar for children and adolescents. One study showed that yogurt was less consumed as a snack by obese children compared to normal weight or overweight children; however, the association was borderline significant ($P = .05$) (Foster et al., 2015).

28.5 HEALTH AND DISEASE

It has long been thought that yogurt is a probiotic food; however, only yogurts made with specific probiotic strains can be officially labeled as probiotic yogurt. For example, more than a dozen probiotic species can be added as starter cultures to yogurt in Canada with accepted health claims around digestive tract health such as “provides live microorganisms that contribute to healthy gut flora.” These probiotic cultures are thought to have proven beneficial effects on digestive health (Canadian Food Inspection Agency, 2016). Yogurt made with standard starter cultures, $L. delbrueckii$ ssp. $bulgaricus$ and $S. thermophilus$, has also been thought to confer beneficial health properties to the host that include enhanced immunity. However, purported beneficial effects are thought to be strain specific and, to date, the only legitimate claim recognized by the European Food Standards Agency and the FAO/WHO for standard yogurt cultures is the improvement of lactose digestion for lactose maldigesters (Morelli, 2014). Nevertheless, yogurt contains many nutritive properties that contribute to beneficial health effects of children and adolescents, which extend beyond the probiotic properties of its starter cultures. Yogurt’s unique dairy matrix may be implicated in influencing some of its purported health properties.

28.6 DIGESTIVE HEALTH

The best known health benefit of yogurt is its ability to assist lactose maldigesters break down lactose. Lactose intolerant or lactose maldigester individuals experience gastrointestinal discomfort following the consumption of lactose because of the absence of the lactase enzyme, which breaks down the disaccharide lactose into monosaccharides. Undigested lactose enters the colon and is fermented by colonic bacteria, which can produce gas, bloating, cramping, and diarrhea (Suchy et al., 2010). Yogurt is better tolerated than milk with the same lactose content, primarily because of two mechanisms that assist with lactose digestion in the absence of endogenous lactase: (1) during milk fermentation yogurt bacteria can convert lactose into lactic acid and (2) yogurt bacteria may also have the ability to produce lactase (Adolfsson et al., 2004; Panahi and Tremblay, 2016). Few studies have been conducted with young populations, but these studies are good evidence that yogurt consumption, particularly containing live bacteria, results in fewer gastrointestinal symptoms in lactose-maldigesting children compared to milk (Montes et al., 1995; Shermak et al., 1995). The ability of yogurt to improve lactose digestion makes it a viable alternative to fluid milk, which is extremely important given that lactose intolerance has been linked to osteoporosis incidence, and avoidance of milk products in childhood and adolescence may inhibit peak bone mass accrual (Schiffner et al., 2016).
Yogurt consumption may be involved in modulating the gut microbiota by suppressing pathogen growth. A study comparing yogurt consumers (>200 g/day) to nonconsumers showed that the level of Enterobacteriaceae, containing pathogenic bacteria, was significantly lower in yogurt consumers, and *Bifidobacterium*, beneficial bacteria, was positively correlated to fermented milk intake. Furthermore, yogurt culture *L. delbrueckii* ssp. *bulgaricus* survived passage through the digestive system and was detected in sufficient quantities to exert biological effects (Alvaro et al., 2007). Another study showed that the consumption of both heat-treated and fresh yogurts was associated with increased lactic acid bacteria and *Clostridium perfringens* and a decrease in *Bacteroides*, providing evidence of positive changes in human microbiota following yogurt consumption (Garcia-Albiach et al., 2008). Yogurt contains lactic acid bacteria, which are thought to regulate gut immune function through antimicrobial activities. Lactic acid bacteria reduce the pH of the gut, produce antimicrobial compounds (e.g., bacteriocins), and compete with pathogens for nutrients and space in the gut (Adolfsson et al., 2004). The ability of yogurt to contribute to pathogenic bacteria suppression can potentially reduce susceptibility to childhood infections. This potential for yogurt to modulate or normalize the microbiota in children can provide promising therapies for undernutrition associated with disrupted microbiota development. Dietary ingredients promoting the development of the gut microbiota are currently under investigation (Blanton et al., 2016). Gut microbiota changes have also been observed in obesity, type 2 diabetes, and irritable bowel syndrome. While 60%–70% of the microbiota is established in the first 3 years of life and remains unchanged throughout life, another 30%–40% can be modified throughout childhood and adulthood, and diet is a potent modulator (Kashtanova et al., 2016).

Diarrhea, defined as the frequent passage of watery bowel movements, is a leading cause of morbidity and death from dehydration and malnutrition in children, particularly young children. The WHO recommends treating diarrhea in children with foods that are familiar and suitable for their age. Salted yogurt drinks can be provided to help recover from dehydration. Additionally, yogurt can be given in place of milk, if available. Fermented dairy foods or products such as yogurt are recommended because they are easy to digest (WHO, 2005). Several studies have found promising results for the use of yogurt in the treatment of acute diarrhea: faster recovery (Niv et al., 1963), better weight gain (Bhatnagar et al., 1998), decreased stool frequency (Boudraa et al., 2001; Van Niel et al., 2002), and reduced duration of hospitalization (Pashapour and Iou, 2006). During episodes of persistent diarrhea, milk can be substituted with yogurt to reduce lactose malabsorption (Dewit et al., 1987). In randomized clinical trials, yogurt appears to be effective in reducing weight loss and the duration of persistent diarrhea (Touhami et al., 1992). It has been postulated that yogurt consumption may help prevent antibiotic-associated diarrhea through modulation of the gut microbiota; however, there are too few studies and low-quality data to confirm the hypothesis. In a metaanalysis, only two randomized controlled trials were identified and no significant reduction in risk of antibiotic-associated diarrhea was noted (Patrógolab et al., 2015). Yogurt may also be an effective method for the delivery of probiotics, providing nutrition in addition to gut restorative bacteria (Fox et al., 2015).

### 28.7 CARDIOMETABOLIC HEALTH

Various mechanisms can potentially protect children and adolescents who consume yogurt from the development of major cardiometabolic diseases later in life such as being overweight and obese, type 2 diabetes, hypertension, cardiovascular disease, and the metabolic syndrome. These mechanisms are related to the nutritive properties of yogurt and its ferments; the positive relationship between yogurt
consumption and healthier lifestyles, behaviors, and dietary patterns; and the substitution or replacement of nutrient-rich snacks with energy-dense, nutrient-poor snacks. Eating behaviors and preferences established in childhood persist in adulthood (Russell et al., 2016). Poor dietary behaviors developed at an early age are likely to be carried through to adulthood and are important factors associated with the development of obesity and associated diseases (Marette and Picard-Deland, 2014). This makes childhood an important life period to study, and during which interventions regarding the introduction and promotion of nutritious foods are critical. In young populations there are also opportunities to intervene at both school and family settings (Ebbeling et al., 2002). Consumers of yogurt often have higher fruit and vegetable intakes (Wang et al., 2013), which is a positive and protective dietary behavior linked to lower incidence of diet-related diseases. The link between yogurt intake and fruit intake may be related to consumption of the two foods in combination (e.g., smoothies), which also offers the potential for synergistic cardiometabolic impacts of two foods with prebiotic and probiotic properties (Fernandez and Marette, 2017).

Yogurt has been identified as a food that can help maintain weight in adult populations in prospective cohort studies (Mozaffarian et al., 2011). Increasing calcium intake in the form of two cups a day of yogurt has been tested in randomized clinical trials and been shown to be effective in promoting weight loss among African-American obese women (Zemel et al., 2000). Isocaloric substitution of foods with yogurt increased fat loss and central adiposity during an energy-restricted diet, a relationship that appears to be mediated by the calcium content of yogurt (Zemel et al., 2005). Low intake of calcium is thought to be involved in promoting positive energy and obesity. Given that dairy intake is a major contributor to dietary calcium and intakes are typically lower than recommended, it would be reasonable to assume that consumption of dairy products such as yogurt can contribute to energy balance (Panahi and Tremblay, 2016), particularly in the context of snack substitution. In populations where frequent snacking of poor quality snacks accounts for a large proportion of daily caloric intake (Larson and Story, 2013), it would be advantageous to promote yogurt consumption in place of nonnutritious alternatives to prevent excessive caloric intake. Yogurt in combination with fruit or vegetables has been suggested as a health-promoting snack that can help children in the United States meet recommendations for nutrients of concern in addition to developing important habits promoting weight maintenance (Hess and Slavin, 2014).

Few studies have examined yogurt consumption separately from intake of total dairy products in relation to childhood weight status or indices of metabolic health, and to our knowledge no studies have tested the effects of substituting yogurt with other snacks on children’s health parameters. An American study using data from 3761 children (5–11 years) and 1803 adolescents (12–16 years) from the third National Health and Nutrition Examination Survey (NHANES III) stated that yogurt consumption was consumed by too few subjects to evaluate its effects on adiposity independently from other dairy products (Bradlee et al., 2010). One modeling study in the United Kingdom examined the change in nutrient intakes after adding 125 g of low-fat fruit yogurt to diets of adolescents. Increases in micronutrients were noted, particularly for calcium; however, there were simultaneous increases in energy and added sugar intake. In the absence of food substitutions, the potential impact of yogurt supplementation on energy balance in this population is unknown (Williams et al., 2015). A study comparing the differences between low yogurt intake (equal or below median) and appropriate yogurt intake (above median) in a cross-section of Azorean adolescents did not find any differences in weight, percent body fat, waist circumference, or weight status between the groups. Furthermore, there were no differences in metabolic parameters [blood pressure, cholesterol, triglycerides, fasting glucose, fasting insulin, and homeostatic model assessment (HOMA)] between the two groups. Using the median intake as a cut-off may not have been an appropriate
method to separate groups, and intakes of consumers versus nonconsumers may have been a more meaningful way to compare data (Abreu et al., 2014). Using NHANES data from 2005 to 2008 from 3876 American children aged 8–18 it was found that yogurt consumers had significantly lower waist circumferences and BMI than nonyogurt consumers. These children also had lower intakes of saturated fat and higher intakes of calcium, vitamin D, and potassium (Keast et al., 2015). In a multicountry study involving a cross-section of 3528 adolescents (12.5–17.5 years) from eight European cities in the HELENA study the relationship between cardiometabolic risk and dairy consumption was examined. Consumption of the yogurt group (yogurt, milk-based beverages, and yogurt-based beverages) was inversely associated with waist circumference and positively associated with cardiorespiratory fitness in both sexes. Additionally, in girls, the yogurt group was inversely associated with BMI z-score, sum of four skin-folds and cardiovascular risk score. However, after controlling for multiple covariates, the relationship only remained significant for waist circumference and sum of skin folds in girls (Bel-Serrat et al., 2014). In another study using data from NHANES (2003–06) with a cross-section of 5124 American children 2–18 years the metabolic profiles of frequent yogurt consumers (≥1/w) were examined. Frequent yogurt consumers had lower fasting insulin levels, lower HOMA insulin resistance index, and greater insulin sensitivity. In this group, yogurt consumption was associated with a higher healthy eating index, but not with body weight, fasting glucose, lipid profiles, blood pressure, or C-reactive protein (Zhu et al., 2015). Promising results were seen in an Australian study examining the association between dairy foods and retinal microcirculation in a cross-sectional analysis of adolescents. Adolescents with the highest tertile of yogurt consumption had healthier retinal microvascular profiles compared to the group with the lowest tertile of yogurt consumption, which is an indication of future healthy vascular profiles (Gopinath et al., 2014). All the studies that have been described show a variety of age groups and cardiovascular health indicators. There are too few studies and too much variation to compare and draw any definite conclusions. It can be hypothesized, however, that given the neutral and positive findings presented between yogurt consumption and cardiometabolic health indices, there is reasonable evidence to pursue targeted studies that investigate the suspected health benefits of yogurt in children and adolescents. Prospective studies are recommended to get a better understanding of long-term effects of yogurt consumption from childhood to adolescence to adulthood on metabolic parameters and diet-related disease development.

28.8 CONCLUSIONS AND FUTURE RESEARCH

At the moment, most of the research on children’s diet quality and dairy consumption trends in relation to nutrition adequacy, dairy product intake, and health has originated from the United States and occasionally from the United Kingdom. There are emerging publications from non-Western populations such as Brazil; however, the limited literature on the topic makes it difficult to generalize findings to other populations given the cultural differences in eating behaviors and preferences as well as the differences in focus of nutrient of children’s needs in developed compared to developing nations, where malnutrition is still a problem. Nevertheless, yogurt appears to be a universally culturally acceptable food that is relatively accessible. It provides children and adolescents with a variety of nutrients that can contribute to healthy eating patterns and adequate nutrition status promoting long-term health. The majority of studies conducted to date have been cross-sectional and there is little known about the impact of yogurt consumption in youth on adult disease development. Future research should look into the behavioral aspects and health implications of long-term yogurt consumption in younger
populations and the persistence of yogurt consumption behaviors into adulthood. The effects of yogurt on gut microbiota and the subsequent implications for protecting against gastrointestinal and cardiometabolic diseases are definite additional avenues for future research.

REFERENCES


REFERENCES


Chapter 28: Role of Yogurt in the Nutrition and Health


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YOGURT CONSUMPTION AND IMPACT ON BONE HEALTH

29

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29.1 INTRODUCTION
Fermented dairy products, particularly cheese, have been used for thousands of years to preserve milk, to make it more transportable, less perishable, readily available, and more digestible, because of lactose breakdown during the fermentation process. This processing of milk was an important development in early agriculture, which can be dated back to the sixth millennium BC in Northern Europe (Salque et al., 2013). Through their content in calcium, phosphorus, proteins, and micro-nutrients, dairy products play a role in the control of bone homeostasis (Table 29.1) (Rizzoli, 2014). Fermented milk products like yogurts may provide larger amounts of these nutrients than the same volume of plain milk because of enrichment with milk powder to make the yogurt matrix denser. Prebiotics like inulin, which may be added to yogurt to also increase matrix density, as well as probiotics, can influence intestinal calcium absorption and/or bone metabolism (Weaver, 2015). Finally, yogurt consumption may ensure a more regular ingestion of milk products because of various flavors and sweetness.

29.2 BONE HOMEOSTASIS
At a given age, bone mass is determined by the amount of bone accumulated at the end of skeletal growth, the so-called peak bone mass, and by the amount of bone lost subsequently (Rizzoli et al., 2010). There is a large body of evidence linking nutritional intakes, particularly calcium and protein, to bone growth, and to bone loss later in life, both influencing fracture risk. Optimal dietary calcium and protein intakes are necessary for bone homeostasis during growth as well as in the elderly. Dairy products may represent the best dietary sources of calcium due to the high content, high absorptive rate, and relatively low cost (Rizzoli, 2014). For example, 250 mg of calcium may be obtained from a 200 mL glass of milk, a 180 g serving of yogurt, or 30 g of hard cheese. To attain from other dietary sources a calcium supply equivalent to one serving of dairy, 5–6 servings of vegetables (dark green leaves or legumes) or 10–12 servings of whole grain or refined grain foods would be required. Dairy products may represent up to 52%–65% of the RDI of calcium and 20%–28% of the protein requirement (Rizzoli, 2014).
29.3 BONE MASS ACCRUAL: ROLE OF DIETARY CALCIUM AND PROTEIN

Peak bone mass is achieved for most parts of the skeleton by the end of the second decade of life (Rizzoli et al., 2010). Body mineral mass nearly doubles during puberty, through an increase in the size of the skeleton, with minor changes in volumetric bone density. Puberty is the period during which the sex difference in bone mass observed in adult subjects becomes fully expressed. This gender difference in bone mass mainly results from a greater increase in bone size (Seeman, 2003). It is estimated that a 10% increase in peak bone mass could reduce the risk of osteoporotic fractures during adult life by 50%, or to be equivalent to a 14-year delay in the occurrence of menopause (Hernandez et al., 2003). A recent evidence-based review of the literature since 2000 of factors influencing peak bone mass development concludes that good evidence is available for a positive role of dairy consumption, whilst the best evidence is for positive effects of calcium intake and physical activity (Weaver et al., 2016). In addition, a systematic review of observational studies that examined the association between dietary intake and childhood fractures concludes that fracture risk appears to be associated with milk avoidance (Handel et al., 2015).

29.3.1 CALCIUM AND BONE MASS ACCRUAL

Calcium plays major roles in the regulation of various cell functions, in the central and peripheral nervous systems, in muscle, and in exo-/endocrine gland function (Rizzoli, 2008). In addition, this cation is implicated in the process of bone mineralization, by the formation of hydroxyapatite crystals.

Several prospective randomized, double-blind, placebo-controlled intervention trials have concluded that calcium supplements increase bone mass gain, although the magnitude of the calcium

Table 29.1 Nutrients Content per 100 g

<table>
<thead>
<tr>
<th>Source</th>
<th>Calcium (mg)</th>
<th>Phosphorus (mg)</th>
<th>Protein (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Milk, 3.7% fat</td>
<td>119</td>
<td>93</td>
<td>3.3</td>
</tr>
<tr>
<td>Milk, skimmed</td>
<td>122</td>
<td>101</td>
<td>3.4</td>
</tr>
<tr>
<td>Yogurt, plain low fat</td>
<td>183</td>
<td>144</td>
<td>5.3</td>
</tr>
<tr>
<td>Yogurt, fruit low fat</td>
<td>169</td>
<td>133</td>
<td>4.9</td>
</tr>
<tr>
<td>Parmesan cheese</td>
<td>110</td>
<td>729</td>
<td>38</td>
</tr>
<tr>
<td>Swiss cheese</td>
<td>791</td>
<td>567</td>
<td>26.9</td>
</tr>
<tr>
<td>Cheddar cheese</td>
<td>721</td>
<td>512</td>
<td>24.9</td>
</tr>
<tr>
<td>Cottage cheese</td>
<td>86</td>
<td>190</td>
<td>10.3</td>
</tr>
<tr>
<td>Cream cheese</td>
<td>98</td>
<td>106</td>
<td>5.9</td>
</tr>
<tr>
<td>Ice cream, vanilla</td>
<td>128</td>
<td>105</td>
<td>3.5</td>
</tr>
</tbody>
</table>

Depending on the preparation (no addition of milk powder), yogurt may have calcium, phosphorus, and protein contents similar to plain milk.

effects appears to vary according to the skeletal sites examined, the stage of pubertal maturation at the
time of the intervention, and the spontaneous dietary calcium intake (Bonjour et al., 1997; Chevalley
et al., 2005a,b). The calcium effects could be modulated by an interaction with vitamin D receptor
genotype (Ferrari et al., 1998). The positive effects of calcium supplementation have been ascribed to
a reduction in bone remodeling. Some effects of calcium supplements on bone modeling have also been
described in addition to bone remodeling (Bonjour et al., 1997, 2001a,b; Cadogan et al., 1997; Prentice
et al., 2005). The influence on bone modeling has been illustrated for instance in a double-blind, pla-
ceso-controlled study in which calcium supplementation in prepubertal girls was associated with
changes in projected scanned bone area and in standing height (Bonjour et al., 1997). Thus, calcium
could enhance both the longitudinal and the cross-sectional growth of the bone. When bone mineral
density was measured 7.5 years after the end of calcium supplementation, i.e., in young adult girls, it
appeared that menarche occurred earlier in the calcium-supplemented group, and that persistent effects
of calcium were mostly detectable in those subjects with an earlier puberty (Chevalley et al., 2005a,b).

Most of the studies carried out in children and adolescents have shown that supplementation with
either calcium or dairy foods over 1 to 3 years enhances the rate of bone mineral acquisition, compared
with unsupplemented (or placebo) control groups. In general, these intervention trials increased the
usual calcium intake of the supplemented children from about 600–800 mg/day to around 1000–
1300 mg/day. In a meta-analysis of 19 calcium intervention studies involving 2859 children, with doses
of calcium supplementation varying between 300 and 1200 mg/day, and various sources of calcium
(from calcium citrate-malate, calcium carbonate, calcium phosphate, calcium lactate-glucurate, cal-
cium phosphate milk extract, or milk minerals), standardized mean differences (effect size) of calcium
supplementation was 0.14 for whole body bone mineral content and upper limb bone mineral density
(Winzenberg et al., 2006). At the upper limb, the effect persisted up to 18 months after cessation of
calcium supplementation.

### 29.3.2 DIETARY PROTEIN AND BONE MASS ACCRUAL

In children and adolescents, protein intakes influence bone growth and bone mass accumulation
(Rizzoli et al., 2010). In “well” nourished children and adolescents, variations in the protein intake
within the “normal” range can have a significant effect on skeletal growth and thereby modulate the
 genetic potential in peak bone mass attainment. Bone mineral density (BMD)/bone mineral content
(BMC) changes in prepubertal boys are positively associated with spontaneous protein intake (Rizzoli
et al., 2010). Furthermore, higher protein intakes interact with the positive influence of physical activity
on proximal femur BMD in prepubertal boys (Chevalley et al., 2008) and also on bone microstructure
in postpubertal boys (Chevalley et al., 2014) and in young adult males (Chevalley et al., 2016). Nutritional environmental factors appear to affect bone accumulation at specific periods during infancy
and adolescence. In a prospective cohort of female and male adolescents aged 9–19 years, we found a
positive correlation between lumbar and femoral bone mass yearly gain and calcium or protein intake
(Bonjour et al., 2001a,b). This correlation was mainly detectable in prepubertal adolescents but not in
those having reached a peri- or postpubertal stage. It remained statistically significant after adjustment
for spontaneous calcium intakes. In another prospective longitudinal study in healthy children and
adolescents of both genders, between the ages of 6 and 18, a positive association was found between
long-term protein intakes, on one hand, and periosteal circumferences, cortical area, bone mineral con-
tent, and with a calculated strength strain index, on the other hand (Alexy et al., 2005). In this cohort
with a Western-style diet, protein intakes were around 2 g/kg body weight per day in prepubertal children, whereas they were around 1.5 g/kg per day in pubertal individuals. Overall, protein intakes accounted for 3%–4% of the bone parameters variance.

It is quite possible that protein intake could be to a large extent related to growth requirement during childhood and adolescence. Only intervention studies could reliably address this question. To our knowledge, there is no large randomized controlled trial having specifically tested the effects of dietary protein supplements on bone mass accumulation, except for milk or dairy products.

In addition to calcium, phosphorus, calories, and vitamins, 1 L of milk provides 32–35 g of proteins, mostly casein, but also whey proteins, which contain numerous growth-promoting elements. In growing children, long-term milk avoidance is associated with smaller stature and lower bone mineral mass, either at specific sites or at the whole body levels (Opotowsky and Bilezikian, 2003). Low milk intake during childhood and/or adolescence increases the risk of fracture before puberty (a 2.6-fold higher risk has been reported), and possibly later in life (Goulding et al., 2004). In a 7-year observational study, there was a positive influence of dairy product consumption on bone mineral density at the spine, hip, and forearm in adolescents, leading thereby to a higher peak bone mass (Matkovic et al., 2004). In this study, higher dairy product intakes were associated with greater total and cortical proximal radius cross-sectional area. Whereas calcium supplements could influence volumetric BMD, thus the remodeling process, dairy products may have an additional effect on bone growth and periosteal bone expansion, i.e., an influence on modeling. In agreement with this observation, milk consumption frequency and milk intake at ages 5–12 and 13–17 years were significant predictors of the height of 12–18 year-old adolescents, studied in the National Health and Nutrition Examination Survey 1999–2002 (Wiley, 2005).

The earliest milk intervention controlled studies are by Orr (1928) and Leighton and Clark (1929). In British schoolchildren, 400–600 mL/day of milk had a positive effect on height gain over a 7-month period. Numerous intervention trials have demonstrated a favorable influence of dairy products on bone health during childhood and adolescence (Cadogan et al., 1997; Cheng et al., 2005). In an open randomized intervention-controlled trial, 568 mL/day milk supplement for 18 months in 12-year-old girls (Cadogan et al., 1997) provided an additional 420 mg/day calcium and 14 g/day protein intakes. In the milk-supplemented group, serum IGF-1 levels were 17% significantly higher. Compared to the control group, the intervention group had greater increases of whole body bone mineral density and bone mineral content.

In another study, cheese supplements appeared to be more beneficial for cortical bone accrual than a similar amount of calcium supplied in the form of tablets (Cheng et al., 2005). The positive influence of milk on cortical bone thickness may be related to an effect on the modeling process, since metacarpal periosteal diameter was significantly increased in Chinese children receiving milk supplements (Zhu et al., 2005).

### 29.4 AGE-ASSOCIATED BONE LOSS AND FRACTURES: ROLE OF CALCIUM AND PROTEIN

#### 29.4.1 CALCIUM

Calcium and vitamin D supplements have been reported to reduce nonvertebral and hip fracture risk in older people (Chapuy et al., 1992). Two subsequently published large trials have challenged these
conclusions by being unable to detect significant antifracture effect in calcium- and vitamin D-treated individuals (Grant et al., 2005; Jackson et al., 2006). Neither study has targeted individuals at high fracture risk, and in both the adherence was poor. The clinical trial of the Women’s Health Initiative was carried out in healthy postmenopausal women with an average calcium intake above 1000 mg/day, 80% of whom were under 70 years of age. When the analysis was carried out in only the compliant subjects, a significant (29%) reduction in hip fracture risk compared to the placebo group was found (Jackson et al., 2006).

A meta-analysis of nine randomized clinical trials, including a total of 53,260 patients, found that supplementation with vitamin D alone was not sufficient to significantly reduce the risk of hip fracture in postmenopausal women, whereas combined supplementation with vitamin D and calcium reduced the risk of hip fracture by 28% and the risk of nonvertebral fracture by 23% compared to supplementation with vitamin D alone (Boonen et al., 2007). Calcium supplements may be associated with mild gastrointestinal disturbances such as constipation, flatulence, nausea, gastric pain, and diarrhea. Calcium may also interfere with the intestinal absorption of iron and zinc. Recently it has been reported that calcium supplementation in healthy postmenopausal women was associated with an increased risk of cardiovascular events (Bolland et al., 2010), mainly in those with a high spontaneous calcium intake. The risk was not affected if calcium was from dietary origin.

An expert consensus meeting of the European Society for Clinical and Economic Aspects of Osteoporosis, Osteoarthritis and Musculoskeletal Diseases and the International Foundation for Osteoporosis reanalyzed the benefit/risk ratio of calcium–vitamin D supplementation and came to the following conclusions (Harvey et al., 2016): (1) calcium and vitamin D lead to a modest reduction in fracture risk; (2) there is no argument for providing calcium alone; (3) side effects of calcium supplementation include renal stones and gastrointestinal symptoms; (4) vitamin D rather than calcium may reduce fall risk; and (5) there is no current convincing evidence for an increased cardiovascular risk. They conclude that calcium–vitamin D supplementation can be recommended to subjects at high risk of calcium and vitamin D insufficiencies and in patients receiving antiosteoporosis pharmacological treatments.

### 29.4.2 PROTEIN

 Virtually all studies assessing a possible association between bone mass at various skeletal sites and spontaneous protein have found a positive relationship in children or adolescents (Chevalley et al., 2008; Alexy et al., 2005), in pre- or postmenopausal women, and in men (Darling et al., 2009). Unadjusted BMD was greater in the group with the higher protein intake in a large series of data collected in the frame of the Study of Osteoprotic Fracture (Sellmeyer et al., 2001). Dietary protein accounted for as much as 2% of bone mineral mass variance. A longitudinal follow-up in the frame of the Framingham study has demonstrated that the rate of bone mineral loss was inversely correlated to dietary protein intake (Hannan et al., 2000), and the risk of hip fracture tended to be lower in older adults with greater intakes of milk and milk + yogurt. The association with fracture risk was only partially explained by the effects on BMD (Sahni et al., 2014). In an analysis of dietary protein food clusters among middle-aged and older men and women, the same group found that BMD was higher in the low-fat milk cluster compared to the red meat protein food cluster and processed foods protein cluster (Mangano et al., 2015). In the large Nurses’ Health Study, a trend for a hip fracture incidence inversely related to protein intake has been reported (Feskanich et al., 1997). The same
study reported an increase of forearm fracture risk in the subjects with the highest protein intake of animal origin. In a prospective study carried out on more than 40,000 women, higher protein intake was associated with a reduced risk of hip fracture (Munger et al., 1999). The protective effect was observed with dietary protein of animal origin. In a case–control study, increasing protein intake was associated with a lower hip fracture risk of 65% in the highest quartile in the 50- to 69-year-old age class (Wengreen et al., 2004). In another study, fracture risk was increased when a high protein diet was accompanied by a low calcium intake, in agreement with the requirement of sufficient calcium intake to detect a favorable influence of dietary protein on bone (Meyer et al., 1997; Dargent-Molina et al., 2008).

The review of prospective data examining the link between dairy consumption and fracture risk showed a high heterogeneity between studies, with some positive and negative studies (Rozenberg et al., 2016). It should be noted that the debate regarding the association between dairy intakes and fracture risk mainly came from the data in a cohort of Swedish women. In a meta-analysis of available data in 2010, after exclusion of this cohort, the relative risk of hip fracture for an increase of one glass of total milk intake per day in women was 0.95 (0.90–1.00), \( P = .049 \) (Bischoff-Ferrari et al., 2011). In the cohorts of Swedish women and men, there was a positive association between milk consumption and fracture risk in women (Michäelsson et al., 2014). In contrast, there was a protective effect of soured milk and yogurt intakes on fracture risk, and the positive association between milk consumption and fracture risk was not found in men and was no more observed in women when milk consumption data were analyzed from each repeated questionnaire individually rather than using time updated exposures as in the primary analysis.

Several mechanisms may be involved in the association with protein intake and bone health. A reduction in dietary protein may lead to a decline in calcium absorption and to secondary hyperparathyroidism (Kerstetter et al., 1997, 2005). A low (0.7 g/kg BW), but not a high, protein intake (2.1 g/kg), was associated with an increase in biochemical markers of bone turnover as compared with a diet containing 1.0 g/kg of protein (Kerstetter et al., 1999). It has been claimed that the source of proteins, animal versus vegetal, would differently affect calcium metabolism. This is based on the hypothesis that animal proteins would generate more sulfuric acid from sulfur-containing amino acids than a vegetarian diet. A vegetarian diet with protein derived from grains and legumes may deliver as many millimoles of sulfur per gram proteins as would a purely meat-based diet (Fenton et al., 2011). In the prospective Canadian Multicentre Osteoporosis Study there was a positive association between hip BMD changes and dairy protein intakes, with in parallel a negative association between spine BMD changes and plant protein intakes in postmenopausal women. There was no significant association with nondairy animal proteins intakes (Langsetmo et al., 2015). In this study, low-protein intake (below 15% total energy intake) was associated with increased fracture risk. A cross-sectional study in elderly women showed that peripheral cortical and trabecular bone mass assessed by QCT is positively correlated with dairy intake (Radavelli-Bagatini et al., 2014). Dietary proteins also influence both the production and action of IGF-1, particularly the growth hormone (GH)-insulin-like growth factor (IGF) system (Rizzoli, 2008). In humans, increased intake of aromatic, but not of branched-chain amino acids, is associated with increases in serum IGF-1, intestinal calcium absorption, and 24-h urinary calcium excretion, without any change in biochemical markers of bone turnover (Dawson-Hughes et al., 2007). Dairy products are particularly rich in aromatic amino acids. The restoration of the altered GH-IGF-1 system in the elderly by protein replenishment
is likely to favorably influence not only BMD but also muscle mass and strength, since these two variables are important determinants of the risk of falling. Intervention studies using a simple oral dietary casein-containing preparation that normalizes protein intake improve the clinical outcome after hip fracture and decrease mortality observed at 6 months (Tkatch et al., 1992; Schurch et al., 1998). It should be emphasized that a 20-g protein supplement, as administered in these studies, brought the intake from low to a level still below recommended dietary allowance (0.8 g/kg body weight), avoiding thus the risk of an excess of dietary protein. In these studies, the total length of stay in the orthopedic ward and convalescent hospital was significantly shorter in supplemented patients than in controls. In a multiple regression analysis, baseline IGF-1 concentrations, biceps muscle strength, together with protein supplements accounted for more than 30% of the variance of the length of stay in rehabilitation hospitals ($r^2 = 0.312$, $P < .0005$), which was reduced by 25% in the protein-supplemented group (Schurch et al., 1998). In another controlled trial, dietary protein supplements favorably influenced bone metabolism in the elderly (Hampson et al., 2003). In a short-term study on the kinetics and determinants of the IGF-1 response to protein supplements in a situation associated with low-baseline IGF-1 levels, such as the frail elderly, or patients with a recent hip fracture, we found that a 20 g/day protein supplement derived from dairy foods increased serum IGF-1 and IGF-binding protein-3 already by 1 week, with a maximal response by 2 weeks (Rodondi et al., 2009; Chevalley et al., 2010).

29.5 MICROBIOTA AND BONE: POSSIBLE MECHANISMS

The most abundant cells ($10^{14}$) within the human body are located within the intestinal tract. This number is much higher than the number of cells in human body parenchymes (Huttenhower et al., 2012). These organisms are collectively called the gut microbiota (GM). They mostly refer to the large intestine content, but all parts of the GI tract are colonized with an increase in microorganism concentration from the duodenum to the distal colon. GM is now considered as an organ modulating the expression of genes involved in mucosal barrier function, immune system, food digestion, or energy metabolism as it is capable of fermenting undigested nutrients into short-chain fatty acids (Huttenhower et al., 2012). Various mechanisms have been proposed to link microbiota composition or production to bone metabolism.

29.5.1 INTESTINAL WALL PERMEABILITY

Intestinal wall thickness and surface increases have been reported in relation to changes in microbiota (Trinidad et al., 1999; Mineo et al., 2006), leading to increases in solutes absorption. GM is also involved in digestion and release of dietary nutrients, which are then excreted in urine (Wu, 2014; Ross et al., 2013). Products from fatty acids metabolism, like short-chain fatty acids (acetate, propionate, butyrate) can directly modify local intestinal metabolism (Donohoe et al., 2011; Trinidad et al., 1996), support intestinal barrier function, and can modify bowel content pH, influencing thereby calcium availability and increasing its absorption (Weaver, 2015; Ammann et al., 1988). Since an increase in net intestinal calcium absorption can be achieved by just ingesting more calcium, a change in intestinal absorption is unlikely to account for the effects on bone metabolism.
CHAPTER 29  YOGURT CONSUMPTION AND IMPACT ON BONE HEALTH

29.5.2 ENDOCRINE PATHWAY MODIFICATIONS

An increase in calcium absorption leads to a reduction in PTH production and thus decrease bone resorption. The gut endocrine system could be affected too, as germ-free (GF) animals have a lower serotonin secretion, in relation to reduced tryptophan hydroxylase-1 expression in the large intestine (Sjogren et al., 2012). Serotonin has been shown to lower bone formation (Yadav et al., 2008).

29.5.3 IMMUNE SYSTEM MODULATION

GF animals, hence lacking microbiota, have immature mucosal immune systems (Macpherson and Harris, 2004). CD4 T-cells are decreased like was TNFα and IL6 production (Macpherson and Harris, 2004). GF mice have fewer CD4+ T-cells and germinal centers in the spleen, suggesting that microbiota can modulate immune system development (Macpherson and Harris, 2004). Presence of bioactive TNF alpha is necessary to detect ovariectomy-induced bone loss (Ammann et al., 1997). An association between inflammation and bone loss is well recognized. Depletion of T-cells by anti-CD4 and anti-CD8 antibodies prevents ovariectomy-induced bone loss in mice (Li et al., 2011).

29.6 EVIDENCE FOR A ROLE OF GUT MICROBIOTA IN BONE METABOLISM

In GF mice of the Balb/B6 background, relative bone volume, trabecular number, and cortical area are markedly higher. In animals totally lacking GM, the osteoclasts number is reduced whilst bone formation rate is maintained, suggesting that higher bone mass and microstructure in GF mice is related to a decrease in bone resorption (Sjogren et al., 2012). When recolonized with a normal gut microbiota by 3 weeks of age, mice displayed a reduction in trabecular BMD and cortical area as compared to GF mice, together with higher levels of osteoclast precursors. Supporting a microbiota influence on bone mediated by inflammation-related bone resorption, GF animals have decreased frequency of CD4+ T-cells and CD11b+/GR 1 osteoclast precursors as well as lower bone interleukin-6 and TNFα mRNA expression. These animals displayed lower serotonin levels, but the latter were not changed upon recolonization despite normalization of bone mass, suggesting that serotonin is not the major cause of high bone mass in GF animals (Sjogren et al., 2012).

29.7 PREBIOTICS AND BONE

The prebiotic inulin, which is derived from chicory roots, can be added to yogurt to increase the density of the matrix. Prebiotics are nondigestible fiber compounds that pass undigested through the upper part of the gastrointestinal tract and stimulate the growth and/or activity of bacteria that colonize the large bowel by acting as substrate for them. Prebiotics refer to galacto-oligosaccharides (GOSs), inulin, resistant starch, polydextrose, fructooligosaccharides (FOSs), xylooligosaccharides, and lactulose. Oligosaccharides are composed of 3–10 units of sugars. Their length influences the site of fermentation (Roberfroid et al., 2010). They resemble oligosaccharides occurring naturally in human milk. The mode of action implies the fermentation of fibers within the large intestine leading to the production of short-chain fatty acids such as acetate, propionate, valerate, isovalerate, or butyrate and isobutyrate. The multiple observed changes are an increase of calcium
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bioavailability (Ammann et al., 1988) through a reduction in bowel content pH (Ammann et al., 1988; Weaver et al., 2011), an increase in cecum weight and microvilli surface (Weaver et al., 2011), histone acetylation with epigenetic modulation (Bultman, 2016; Mathewson et al., 2016), as well as microbiota modifications, with an increase in the bifidobacteria species capable of metabolizing phytoestrogens (Matthies et al., 2012). Doses of GOS, FOS, fiber dextrin, inulin, agave fructans, up to 20 g/day, increase the number of bifidobacteria and lactobacilli and decrease that of coliforms. It is also possible that prebiotics have direct effects on the immune system, without metabolism (Bindels et al., 2015).

At the bone level, FOS administration increases cortical and trabecular bone in mice, BMC in male rats (Garcia-Vieyra et al., 2014). Diet enriched in fibers enhances cortical thickness, cortical BMC, and trabecular BMD in rats (Weaver et al., 2011). FOSs are also associated with higher bone strength (Weaver et al., 2011). Regarding bone metabolism, agave fructans increases osteocalcin levels (Garcia-Vieyra et al., 2014), GOS/FOS stimulates osteoblast proliferation (Bryk et al., 2015), and FOS-inulin reduces the ratio bone resorption/bone formation in ovariectomized rats (Zafar et al., 2004). Femur and tibia breaking strength, distal femur total, and trabecular volumetric BMD, as well as proximal tibia volumetric BMD, increased in response to GOS supplementation (Weaver et al., 2011). It has been shown that onions and a mixture of vegetables decreased bone resorption, through a mechanism independent of their supply in alkali (Muhlbauser and Li, 1999; Muhlbauser et al., 2002). Though microbiota changes were not evaluated in these studies, the high-fiber content of these nutrients may have acted as prebiotics.

Human sialylated milk oligosaccharides are less abundant in mother’s milk of severely stunted infants (Charbonneau et al., 2016). Human milk contains various glycans with prebiotic properties contributing to infant immune system development (He et al., 2014). Fermentation products enhance intestinal barrier function by stimulating the assembly of tight junctions (Peng et al., 2009).

In a randomized controlled trial in adolescents, 8 g/day of FOS and inulin for 1 year increased whole body BMC (Abrams et al., 2005). In male adolescents, the consumption of 15 g of oligofructose per day was shown to stimulate fractional calcium absorption (van den Heuvel et al., 1999a,b). Among healthy adolescent girls aged 10–13 years, who consumed smoothie drinks twice daily supplemented or not with GOS for 3-week periods, increase in the fractional calcium absorption was observed compared with nonsupplemented controls (0.444, 0.419 vs. 0.393, respectively). The increase in absorption was greatest after 24 h, consistent with distal gut absorption (Whisner et al., 2013). Whether a small increase in fractional calcium absorption with GOS supplementation may result in a biologically significant increment in bone mineral accrual leading to higher peak bone mass in the long term remains to be demonstrated. This was accompanied by a higher amount of bifidobacteria in the stools (Whisner et al., 2013). Using a similar stable calcium absorption method, this author detected a 12% higher intestinal calcium absorption in adolescent boys and girls exposed to maize and corn fibers (Whisner et al., 2014). Soluble corn fiber increases calcium retention in postmenopausal women (Jakeman et al., 2016).

In various populations of different ages from adolescents to postmenopausal women, and with various treatment durations with prebiotics, from 9 days to 1 year, higher intestinal calcium absorption was consistently detected (Holscher et al., 2015; Griffin et al., 2002; van den Heuvel et al., 1999a,b, 2000; Holloway et al., 2007). The amount of prebiotics to be ingested to produce significant bone effects is limited by the tolerance. Indeed, undigested saccharides/fibers fermentation in the large intestine may be associated with flatulence and abdominal discomfort, precluding amounts of prebiotics ingestion.
sufficient to reach meaningful biological effects. In the studies by Whisner et al. (2016), this issue was addressed, and the tolerance to prebiotics amounts associated with increased calcium absorption was reported as good.

29.8 PROBIOTICS AND BONE

Yogurt contains a starter bacteria consisting of Lactobacillus delbrueckii ssp. bulgaricus and Streptococcus thermophilus, and in probiotic yogurt a number of probiotic bacteria are added in addition to the starter bacteria. Another way to modify GM is to directly provide some bacteria to the GI tract, i.e., probiotics. Probiotics are live microorganisms that, when administered in adequate amounts, confer a health benefit on the host. By adequate, one means an amount able to trigger the targeted effect. It depends on strain specificity, matrix, and sought targeted effect. The concentration is around $10^7$ to $10^8$ probiotic bacteria per gram, with serving size around 100–200 mg. Various species are considered as probiotics, such as Lactobacillus, Bifidobacterium, Escherichia, Enterococcus, and Bacillus subtilis. Yeasts like Saccharomyces have been used too. Probiotics are available in the form of yogurt, milk-based foods, powders, capsules, or solutions like ice cream and beer.

Several studies investigated the influence of probiotics on bone metabolism. Lactobacillus reuteri increases femoral and vertebral BMD in male but not female intact mice (McCabe et al., 2013), prevents ovariectomy-induced bone loss (Britton et al., 2014) and type 1 diabetes–associated osteopenia (Zhang et al., 2015). These latter two models differ by the bone turnover pattern. The former is associated with an increased osteoclastic bone resorption whereas the latter is rather characterized by a low-bone formation. This would suggest that the probiotic L. reuteri is also capable of stimulating osteoblast activity, possibly by preventing TNFα suppression of Wnt10b in bone (Zhang et al., 2015). Lactobacillus paracasei prevents ovariectomy-dependent bone loss (Ohlsson et al., 2014; Chiang and Pan, 2011), together with a decrease in TNFα and IL1β expression in cortical bone. Ovariectomy-induced bone loss is also prevented by Lactobacillus helveticus fermented milk (Narva et al., 2007). The role of probiotics in attenuating bone damage induced by sex hormone deficiency has been extensively studied (Li et al., 2016). In this recent work, hypogonadal GF mice do not show bone loss like hypogonadal mice with intact gut microflora. In GF mice, there is no increase in osteoclasts in response to hypogonadism. When microflora is reintroduced in GF animals, there is a reversal of the osteoprotection observed in the absence of microflora. Estrogen deficiency alters the gut barrier function leading to endotoxemia. This does not occur in GF mice, which in addition do not have an increase in TNF alpha expressing CD4+ T-cells. In this model of hypogonadal mice, the probiotic Lactobacillus rhamnosus prevented estrogen deficiency–mediated bone loss and decreased TNFα and RANKL expression in intestine and bone marrow (Li et al., 2016).

In humans, the main source of probiotics is fermented dairy products. However, with this kind of supply, the specific effects of probiotics as compared to calcium, protein phosphorus, or zinc are difficult to identify. Furthermore, there remains the problem of a sufficient amount of bacteria capable of reaching the distal part of the gastrointestinal tract. However, it has been reported that yogurt consumers had lower level of Enterobacteriaceae and higher beta-galactosidase activity, the latter and Bifidobacterium population being positively correlated to the amount of fermented products ingested (Alvaro et al., 2007). In a 12-year follow-up of the Framingham Offspring Study, yogurt intake was
associated with hip (trochanter) BMD alone. Yogurt intake showed a weak positive protective trend for hip fracture, while no other dairy groups showed a significant association (Sahni et al., 2013). During a median of 22 years, women of the Swedish Mammography Cohort with a high intake of cheese or fermented milk products compared with women with low intakes had lower mortality and fracture rates. For each serving the rate of mortality and hip fractures was reduced by 10%–15% (Michäelsson et al., 2014). In 65-year-old women of the Geneva retiree cohort, yogurt consumers have higher lumbar spine BMD and distal tibia cortical area. Over a 3-year follow-up, women ingesting one or more yogurts per day have attenuated decrease of total hip BMD, distal radius cortical area and thickness, independently of total calcium and protein intakes (Biver et al., 2016).

A number of controlled intervention trials have been conducted in adults testing the effects between fermented dairy product consumption on markers of bone activity. IGF-1 is an essential factor for longitudinal bone growth. IGF-1 can also exert anabolic effects on bone mass during adulthood. Consumption of a vitamin D and calcium-fortified soft cheese by healthy postmenopausal women increases protein intake, reduces the serum concentration of bone resorption biomarkers (TRAP 5b and CTX), and increases serum IGF-1, compatible with a nutrition-induced reduction in postmenopausal bone turnover rate (Bonjour et al., 2012). Similar findings were found in studies on elderly women using soft cheese or yogurt (Bonjour et al., 2009, 2013). Fortifying yogurt with calcium and vitamin D further reduces PTH and bone resorption markers levels (Bonjour et al., 2015). Bedtime consumption of fermented milk reduces nocturnal bone resorption (Adolphi et al., 2009). Supplementation with calcium from milk mineral has no additional effect unless inulin-type fructans and caseinphosphopeptides are added. Urinary calcium excretion increases at a constant bone resorption, suggesting a change in intestinal calcium absorption. Three servings a day of fortified milk and yogurt for 12 months induced more favorable changes in biochemical indexes of bone metabolism and BMD than calcium supplementation alone in postmenopausal women (Manios et al., 2007). Fermented, protein-fortified (12 vs. 3 g), isocaloric dairy product during 4 weeks slightly increase serum IGF-1 levels in young women with anorexia nervosa, without significant changes in bone turnover markers (Trombetti et al., 2016).

From a health economic perspective, there is a potential nutrition economic impact of increased dairy consumption on osteoporotic fractures (Lotters et al., 2013). Similarly, eating one yogurt is cost-effective in the general population above the age of 70 years and in all age groups in women with low BMD or prevalent vertebral fracture. The daily intake of two yogurts is cost-effective above 80 years in the general population and above 70 years in the two groups of women at increased risk of fractures (Ethgen et al., 2016).

A major problem with probiotics administration is certainly that the amount of ingested bacteria is not sufficient to modify gut microbiota composition. Indeed, in adult monozygotic tweens, two servings a day of fermented milk products containing five different species of bacteria did not modify large intestine bacterial species composition. In contrast, when the same fermented milk products were given by gavage to gnotobiotic mice, there was a rapid change (in less than 24 h) in microbiome-encoded enzymes involved in carbohydrate metabolism (McNulty et al., 2011; Rizzoli et al., 2014). In the same study, Bifidobacterium animalis, ssp. lactis upregulated a locus involved in xylooligosaccharides catabolism in both mice and human metatranscriptome.

Some issues remain to be further elucidated with pre- and probiotics. The doses in terms of both efficacy and tolerance are crucial factors. Timing and duration of administration should be further studied, as well as the offset of the effects upon pre- or probiotics discontinuation.
29.9 CONCLUSION

Besides its effects on body weight or control of type 2 diabetes, yogurt consumption positively influences bone growth and bone homeostasis through different mechanisms involving intakes of key nutrients such as calcium, phosphorus, and proteins, as well as potentially pre- and probiotics. In this respect, gut microbiota may be implicated. Bone mass accrual, bone homeostasis, and attenuation of sex hormone deficiency-induced bone loss seem to benefit from pre- or probiotics ingestion, which modifies microbiota composition and metabolism.

REFERENCES


30.1 INTRODUCTION
The HIV epidemic continues to be a leading cause of morbidity and mortality globally, with a higher prevalence in low- and middle-income countries. Investments made in screening, treatment, and prevention have yielded unprecedented results over the past decades. The Millennium Development Goal 6A, to “have halted by 2015 and begun to reverse the spread of HIV/AIDS,” has been achieved. By 2014, the number of people newly infected with HIV declined by 42% and AIDS-related deaths by 40%. There has been good progress to Millennium Development Goal 6B, to “achieve, by 2010, universal access to treatment for HIV/AIDS for all those who need it,” with 14.9 million people living with HIV receiving antiretroviral therapy (ART) in 2014. However, this still represents only 40% of all people living with HIV globally. With the removal of a threshold to initiate ART, it will be a significant challenge to upscale health services providing treatment to all people living with HIV, and to access those who have been “unreached” by treatment programs (WHO, 2015).

HIV has a deleterious impact on a person’s nutritional status. Without access to a balanced diet, the effectiveness of and adherence to ART are reduced. HIV can impair the ability to absorb and utilize nutrients even from a nutritious diet and increases nutritional demands (Beach et al., 1992). Programs aimed at providing access to medical treatment, therefore, also need to be able to provide access to nutritional support (WHO, 2003). Yogurt can be an excellent nutritional intervention for people living with HIV. It can increase the dietary quality and variety, enhance protein and energy intake, and supplement the nutrients depleted by HIV. Moreover, yogurt can act as an excellent vector for probiotics, prebiotics, and micronutrients with the potential to delay the deterioration of the immune system caused by HIV. This chapter aims to provide the rationale for yogurt as a nutritional intervention for people living with HIV and a vector for delivering pre- and probiotics to reduce the progression of HIV.

30.2 HIV AND THE GASTROINTESTINAL TRACT
Early in the course of HIV the virus preferentially targets CD4+ T-cells associated with the mucosal immune system, resulting in massive loss of gut-associated CD4+ T-cells and an anatomical and functional alteration of the intestinal epithelium (Brenchley et al., 2004). This leads to dysfunction of the mucosal barrier resulting in leakage of bacterial products including bacterial lipopolysaccharide (LPS),
a component of Gram-negative bacterial cell membranes (Brenchley et al., 2006). LPS and other bacterial products are potent inflammatory agents leading to a release of proinflammatory cytokines such as interleukin-6, ultimately resulting in systemic immune activation (Deeks et al., 2013). In this inflammatory state, HIV replication is markedly hastened leading to disease progression (Marchetti et al., 2011). Studies have demonstrated that despite effective ART, the mucosal immune system cannot be entirely restored and bacterial translocation continues not only to support ongoing HIV replication but is also associated with non-AIDS-associated disorders such as progression of atherosclerosis and increased cardiovascular risk, nonalcoholic fatty liver disease, and metabolic syndrome (Deeks et al., 2013).

Impairment of the mucosal immune system may also result in the significant intestinal dysbiosis observed among people living with HIV. Multiple studies have shown increased fractions of opportunistic bacteria and reduced fractions of potentially beneficial *Bifidobacterium* and *Lactobacillus* species in the intestinal microbiome among people living with HIV (Gori et al., 2008). Dysbiosis has been directly associated with increased levels of LPS and systemic immune activation (Pérez-Santiago et al., 2013). Despite ART, dysbiosis is not restored to normal; this strengthens the rational for additional gut-altering treatments (Mutlu et al., 2014).

Counteracting the mucosal changes caused by HIV and targeting the associated impaired microbiome is where yogurt itself or as vector for prebiotics and probiotics can potentially play a role in improving the mucosal barrier and reducing the ongoing inflammatory state of people living with HIV. This may not only have an effect on HIV disease progression but may also impact non-AIDS-associated disorders and the related morbidity and mortality.

### 30.3 HIV AND NUTRITION

Before AIDS had a name, the condition was simply called “slim disease,” marking the evident impact of HIV on a person’s nutritional status. Patients with untreated HIV have an increased resting energy expenditure of 10% (Kosmiski, 2011), a reduced appetite, impaired digestion, and malabsorption of nutrients. Protein metabolism can be increased by 25% in untreated people, frequently associated with muscle mass wasting (Macallan et al., 1995). In addition, among people living with HIV, micronutrient deficiencies are common and thought to be associated with increased metabolic requirements, enhanced excretion, and intestinal malabsorption (Beach et al., 1992). Optimal immune function is dependent on availability of macro- and micronutrients. In HIV, where patients often already have an impaired nutritional status before infection, the absence of a nutritionally adequate diet is a major contributor to an impaired immune function, progression of HIV, and mortality. Indeed, weight loss or a low body mass index prior to the initiation of ART is an independent risk factor for treatment failure and mortality (Koethe and Heimburger, 2010). Despite the development of effective ART, people living with HIV continue to be at increased risk of nutritional disorders (Wanke et al., 2000). The World Health Organization acknowledges that an adequate diet is essential in the clinical care of patients and achieving optimal nutrition is believed to improve an individual’s immune function, delay progression to AIDS, and reduce the incidence of HIV-related illnesses (WHO, 2003). Yogurt as a palatable food, with a high content of easily digestible protein and essential micronutrients, can potentially act as a safe adjunct to nutritional interventions among people living with HIV.
30.4 YOGURT AS A NUTRITIONAL INTERVENTION IN HIV

An ideal nutritional intervention for people living with HIV would be a palatable product that contains good quality protein and essential micronutrients and can enhance a protective intestinal microbiome. Moreover, it should be an economically sustainable product that is not easily contaminated and can be locally produced.

Yogurt does fulfill the majority of the above criteria. Especially in low-income countries where diets frequently have a large proportion of low-nutrient-dense starches, yogurt can add significant nutritional value. With approximately 15% of the recommendation for protein (WHO, 2007) in a single cup (245 g) of yogurt (USDA, 2016), it is a high-quality means of increasing dietary protein and essential amino acids, which are necessary to support adequate immune function. Yogurt is also a source of bioavailable vitamins that are essential in HIV. Trials with supplementation of vitamin B2 (riboflavin), vitamin B6 (pyridoxine), and vitamins B12 (cobalamin), C, E, and folic acid have shown to reduce the progression of HIV (Hummelen et al., 2010). A cup of yogurt contains 27% of the recommended nutrient intakes (RNIs) (WHO, 2007) of vitamin B2, 6% of vitamin B6, 38% of vitamin B12, 3% of vitamin C, and 4% of folate. In addition, serum concentrations of zinc and selenium are frequently low among people living with HIV and are associated with an impaired immune function. Yogurt provides 24% and 18%, respectively, of the RNI (assuming moderate zinc bioavailability) of zinc and selenium. As such, yogurt is a protein and micronutrient-dense food suitable for people living with HIV.

The natural nutritional value of yogurt can be complemented by adding specific micronutrients. Milk products in developed countries are frequently enriched with vitamin A. Hemsworth et al. (2012) developed a probiotic yogurt with added micronutrients, specific to the requirements of people living with HIV, which was both palatable and feasible to produce on a smaller scale. Adding local nutrient-dense foods to yogurt has been shown to be feasible in Tanzania, where Moringa leaves supplemented locally produced probiotic yogurt, significantly increasing the content of vitamin A (Van Tienen et al., 2011). The advantage of adding micronutrients to a yogurt product is that absorption and availability can be enhanced. Therefore, yogurt in itself is an excellent source of essential amino acids, protein, vitamins, and minerals essential for maintaining the immune function of people living with HIV, but can also relatively easily be supplemented with locally sourced or industrially available micronutrients.

Yogurt is the result of milk fermentation by Lactobacillus delbrueckii ssp. bulgaricus and Streptococcus thermophilus. Even though these species do not have inherent probiotic properties, their presence may improve the balance and the metabolic activities of the indigenous intestinal microbiota (Pei et al., 2015). In addition, among the general population, dairy products have been associated with an increase in bone mineral density and a reduced risk of metabolic syndrome (van Meijl et al., 2008). Among people living with HIV, with an increased risk of osteoporosis, cardiovascular disease, and nonalcoholic fatty liver disease, yogurt could have an even greater beneficial effect.

Globally, and in particular in the African region, yogurt is a product that is popularly used and generally accepted. Even though the majority of the world’s population is lactose intolerant, bacteria used for fermentation can hydrolyze lactose into easily digested simple carbohydrates, glucose and galactose, reducing the symptoms associated with lactose intolerance. The natural acidity of yogurt increases the shelf life of the product and prevents contamination with pathogens. The production of smaller quantities of probiotic yogurt using a simple process has proven to be feasible and is currently being done in Bangladesh, Rwanda, Kenya, and Tanzania on an increasingly large scale. The Western Heads East initiative, Yoba for Life, and the Grameen bank have used these principles to set up yogurt
kitchens, often with a probiotic strain, to produce affordable yogurt for children and adults with HIV. Observational studies have shown that the supplementation of a probiotic yogurt is associated with an increase in CD4 count (Irvine et al., 2010).

30.5 YOGURT AS A VECTOR FOR PROBIOTICS IN HIV

Probiotics are defined as “live microorganisms which when administered in adequate amounts confer a health benefit on the host” (WHO, 2003). Probiotics are frequently administered in yogurt as yogurt can buffer stomach acid and bile. In addition, yogurt is an excellent delivery system for probiotics in low-resource communities where more advanced technology is frequently unavailable. The following section focuses mainly on the potential of probiotics to reduce the progression of HIV, with yogurt as a potential delivery system for specific probiotic strains.

In specific patient groups, probiotic strains have shown to reduce microbial translocation and improve the gut barrier function (Liu et al., 2013), downregulate inflammation (Vaghef-Mehrabany et al., 2014), and ameliorate gastrointestinal symptoms. In an animal model of HIV, probiotic and prebiotic supplementation were shown to enhance reconstitution of CD4+ T-cells and reduce the occurrence of functional changes in the gastrointestinal tract (Klatt et al., 2013). Based on these findings, approximately 20 studies have been carried out to assess whether probiotics can reduce gastrointestinal symptoms, progression of HIV, and, more recently, microbial translocation and measures of systemic inflammation. One of the first studies of probiotics among people living with HIV was reported by Salminen et al. (2004) who conducted a small randomized controlled trial (RCT) to assess the effect of Lactobacillus rhamnosus GG on ART-induced gastrointestinal symptoms. The study reported negative findings with no change in CD4 count and viral load. Shortly after, a RCT by Trois et al. (2008) among children with HIV in Brazil (n=77) and an RCT by Anukam et al. (2008) in Nigeria among adult women with HIV (n=24) reported a significant increase in CD4 count. Larger RCTs in Tanzania among ART naive adults with HIV could not replicate the findings of an increase in CD4 count with up to 25 weeks of follow-up (Hummelen et al., 2011a,b). In addition, a randomized crossover trial in Canada on supplementation of L. rhamnosus GR-1 among HIV patients receiving ART also did not report an increase in CD4 count (Hemsworth et al., 2012). A recent RCT by Yang et al. (2014) among 24 patients with HIV treated with ART showed an increase in relative CD4 count among the treatment group supplemented with Bacillus coagulans, although there was no change in absolute CD4 count. These results are contrasted with an observational study in Tanzania that showed that consuming yogurt with L. rhamnosus GR-1 was significantly associated with an increase in CD4 count in adults both treated and untreated with ART (Irvine et al., 2010). Possibly the longer follow-up of this observational study (3 years) and the larger group made it possible to detect a meaningful change in CD4 count.

Studies on the effect of probiotic supplementation on HIV started yielding more conclusively positive results when measures of bacterial translocation, such as soluble CD14 and LPS-binding protein (both indirect measures of lipopolysaccharide in serum), and their effects on the immune system, such as CD4 T-cell activation, started to be taken into account. González-Hernández et al. (2012) were the first to show in an RCT among HIV patients naive to ART that a synbiotic supplement could reduce plasma bacterial DNA and interleukin-6, a potent inflammatory parameter, from baseline. There was, however, no significant difference found in comparison to the control group in this small study (n=20).
Schunter et al. (2012), however, in a similar RCT (n=33), could not show a difference in plasma bacterial DNA concentration or in soluble CD14. In a very well conducted RCT by Villar-García et al. (2015) in 44 adults with HIV on ART, supplementation with Saccharomyces boulardii was shown to significantly reduce the level of LPS-binding protein and IL-6. Supplementation lasted for 12 weeks and no changes in CD4 count were noted. Stiksrud et al. (2015) and d’Ettorre et al. (2015) in RCTs among HIV patients on ART showed a reduction in LPS-binding protein and IL-6 from baseline, with no differences between treatment groups.

The largest RCT reported on HIV and probiotics only has 112 participants, the average follow-up is approximately 12 weeks. Given that RCTs in HIV research usually have several hundred participants and follow-up times of a year or more to show clinical effectiveness, it is not surprising that the results of these RCTs presented are inconclusive. Mechanism-based studies, albeit, have shown a reduction of markers of microbial translocation and inflammation. Though there is limited data available, the probiotic strains S. boulardii (Villar-García et al., 2015), a multistrain probiotic with L. rhamnosus GG, Bifidobacterium lactis BB-12, Lactobacillus acidophilus LA-5 (Stiksrud et al., 2015), and Synbiotic 2000 (González-Hernández et al., 2012; Schunter et al., 2012) appear to have the best evidence as an effective intervention for HIV patients. Potential clinical effectiveness needs to be assessed with a sufficiently powered RCT on clinically meaningful endpoints such as CD4 count, time to start of ART, or morbidity.

A prebiotic is defined as “a selectively fermented ingredient that allows specific changes, both in the composition or activity, in the gastrointestinal microflora that confers benefits upon host well-being and health” (Pineiro et al., 2008). Yogurt can act as a delivery system of prebiotics and also has inherent prebiotic properties. The gut microbiome acts as a first line of defense against opportunistic organisms and plays an essential role in the maintenance of an effective mucosal barrier. Since HIV patients have impaired gut microbiome and mucosal barriers, they could benefit from properties of prebiotics to enhance the mucosal barrier and modulate the gut microbiome.

Among adults with HIV and receiving ART, Gori et al. (2011) conducted a RCT (n=57) to assess the effect of a specific mixture of galacto-oligosaccharides. The study showed that prebiotics reduced the level of soluble CD14, a measure of bacterial translocation, and the percentage of activated CD4+ T-cells, suggesting a reduction in the systemic inflammatory cells. In addition, prebiotic supplementation caused beneficial changes to the gut microbiota including an increase in Bifidobacterium and a reduction in Clostridium species. This pilot study was followed by a seminal RCT by Cahn et al. (2013) among 340 ART-naive individuals with 52 weeks of follow-up. This study showed that subjects within the prebiotic group experienced an average decline of 28 CD4 cells versus a decline of 68 CD4 cells in the placebo group. In addition, the average time to ART initiation was longer in the prebiotic group (230 days) in comparison to the placebo group (167 days). This trial clearly suggests that modulating the intestinal microbiota among HIV patients can have a significant clinical impact. The trial also serves as an example of the types of studies needed within the field of probiotics and prebiotics to bridge the gap from mechanism-based research to clinical applications.
Yogurt has great potential as a nutritional intervention for people living with HIV. Yogurt can significantly improve the protein and nutrient density of low-quality and low-quantity diets; this is required for optimal immune function. Yogurt can be locally produced in resource-poor regions and is accepted in regions most affected by the HIV epidemic. In addition, it can serve as a vector for additional micronutrient, probiotic, and prebiotic supplementation, which have been shown to reduce the sequelae of a proinflammatory state and microbial translocation in HIV.

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Yogurt in Health and Disease Prevention

Edited by Nagendra P. Shah, Professor of Food Science and Technology, School of Biological Sciences, University of Hong Kong, Hong Kong, China

Yogurt in Health and Disease Prevention examines the mechanisms by which yogurt, an important source of micro- and macronutrients, impacts human nutrition, overall health, and disease. Topics covered include yogurt consumption’s impact on overall diet quality, allergic disorders, gastrointestinal tract health, bone health, metabolic syndrome, diabetes, obesity, weight control, metabolism, age-related disorders, and cardiovascular health. Modifications to yogurt are also covered in scientific detail, including altering the protein-to-carbohydrate ratio, adding n-3 fatty acids, phytochemical enhancements, adding whole grains, and supplementing with various micronutrients. Prebiotic, probiotic, and synbiotic yogurt components are also covered to give the reader a comprehensive understanding of the various impacts yogurt and related products can have on human health.

Key Features
• Health coverage encompasses nutrition, gastroenterology, endocrinology, immunology, and cardiology
• Examines novel and unusual yogurts as well as popular and common varieties
• Covers effects on diet, obesity, and weight control
• Outlines common additives to yogurts and their respective effects
• Reviews prebiotics, probiotics, and symbiotic yogurts
• Includes practical information on how yogurt may be modified to improve its nutritive value

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