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CHAPTER 3.5

Bacteria in Food and Beverage Production

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Introduction

Foods are plants and animals (or their products) and each often contain many types of microorganisms. These microbes from natural and external sources contaminate foods by contact, which can occur anytime between production and consumption. Microbial contamination of foods can have many undesirable consequences ranging from spoilage to foodborne illness. However, some microorganisms possess properties that can benefit food production or conversion. Many food-grade microorganisms are used to produce a variety of fermented foods from raw animal and plant materials. The acidic, and in part organolytic, properties of fermented products result from the fermentative activities of these microorganisms. Foods such as ripened cheeses, fermented sausages, sauerkraut and pickles have not only a greatly extended shelf life compared to the raw materials from which they are derived, but also aroma and flavor characteristics contributed directly or indirectly by the fermenting organisms. Consumption of fermented food product has increased dramatically during the last two decades and will continue to increase. The production and availability of fermentative microorganisms (starter cultures) used in food conversion have advanced to meet this demand. This includes the development of novel and better strains through genetic engineering.

Lactic acid bacteria (LAB) are among the most important groups of microorganisms used in food fermentations. LAB contribute to the taste and texture of fermented products and inhibit food spoilage bacteria by producing growth-inhibiting substances (bacteriocins) and large amounts of lactic acid. Many LAB benefit human and animal health, whereas others spoil beer, wine, and processed meats. They can be isolated from the respiratory, intestinal, and genital tracts of humans and animals and from plants.

Fermented dairy products have been made for thousands of years, but only within the last century has the microbiological basis of these fermentations been elucidated. And LAB are the principal organisms involved in fermenting dairy products. Prior to the availability of starter cultures, milk fermentations relied on the LAB naturally present in raw milk. The first commercial starter cultures, unknown mixes of strains most likely from raw milk, were prepared in Denmark just before the end of the 19th century. In the 1930s and 40s, the concept of pure single-strain starter cultures evolved, and a bacteriophage was identified as an important agent of slow fermentation in cheese. With their development in the 1960s and 70s, concentrated (often frozen or freeze-dried) starter cultures could be inoculated directly into the bulk starter vessel without the need for prior preparation in the creamery. In the last two decades, major advances on dairy starter technology have included improved culture selection procedures that enhance bacteriophage resistance. Molecular technology has been applied to map the genetic constructs of starter culture organisms and, by using plasmid/gene transfer mechanisms, to improve starter culture performance.

Sausage is one of the oldest processed meat products. The writings of ancient Egyptians described the preservation of meat by salting and sun drying. The ancient Babylonians, Greeks, and Romans used sausage as a food source during times of war. Microorganisms were recognized as being important to the production of sausages not until about 1921. In the 1940s and 1950s, pure microbial starter cultures became available to processors, and in 1968 frozen culture concentrates became commercially available to the meat industry. Use of these cultures was not widespread until the early 1980s largely because producers clung to the traditional methods of making sausage (using previously fermented meat as the source of LAB) and feared they might lose the quality and consumer acceptance of their final product. Today the importance of the use of starter cultures is recognized in most countries.

The fermentation of vegetables, a practice that originated in the Orient, has been used as a means of preserving food for more than 2,000

years. In the third century B.C. during the construction of the Great Wall of China, the Chinese produced fermented vegetables (cabbages, radishes, turnips, cucumbers, etc.) on a large scale. The most common fermented vegetables available in the United States are pickles, sauerkraut, and olives. Carrots, cauliflower, celery, okra, onions, and sweet and hot peppers also are sold as fermented vegetable products.

Currently, more than 2,000 different fermented foods are consumed by humans worldwide; many are ethnic and produced in small quantities to meet the demand of a group in a particular region. Some are produced commercially, and only a few by large commercial food processors. As consumers' interest in natural and health foods increases, future consumption of fermented foods also will increase significantly worldwide.

Classification of Bacteria in Food and Beverage Products

The classical approach to bacterial classification is based on morphological and physiological features. LAB are Gram-positive, non-sporeforming cocci, coccobacilli, or rods with a DNA base composition of less than 50 mol% G+C (Stiles and Holzapfel, 1997). They generally lack catalase and ferment glucose mainly to lactic acid (homofermentative) or to lactic acid, CO_2, and ethanol or acetic acid or both (heterofermentative). The importance of LAB in the fermentation of food products (dairy, meat, vegetables, fruits, and beverages) has been used as a basis to differentiate the group, although some are also members of normal flora of the mouth, intestine, and vagina of mammals (Klein et al., 1998). Therefore, LAB associated with food are generally restricted to the genera *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus*, and *Weissella*. Table 1 shows differential characteristics of LAB at the genus level (Axelsson, 1998). Bacterial taxonomic classification has been expanded recently to include cell wall composition (de Ambrosini et al., 1996), cellular fatty acids (Gilarova et al., 1994), isoprenoid quinones and other characteristics of cells (Stiles and Holzapfel, 1997). Molecular characteristics also have become important taxonomic tools, such as electrophoretic properties of the gene products, DNA:DNA hybridization studies, and structures and sequence of ribosomal RNA (rRNA; Collins-Thompson et al., 1991; Makela et al., 1992; Stackebrandt and Teuber, 1988; Vandamme et al., 1996). The 16S rRNA data for LAB suggest new groupings that cross the established taxonomic lines. Not all of the new groupings have become established in bacterial taxonomy, but recent phylogenetic considerations indicate that the lactobacilli, leuconostocs and pediococci can be reclassified as three major groups: the *Leuconostoc* group, the *Lactobacillus delbrueckii* group, and the *Lactobacillus casei-Pediococcus* group. The newly established genera *Carnobacteria*, *Tetragenococcus* (previously *Pediococcus halophilus*) and *Vagococcus* (previously motile streptococci) form a phylogenetic cluster with the genus *Enterococcus* (Vandamme et al., 1996). However, generally LAB that are important to food microbiology include only certain species of the genera *Lactobacillus*, *Lactococcus* (*Streptococcus*), *Leuconostoc* and *Pediococcus* (Stiles and Holzapfel, 1997). The genus *Lactobacillus* is by far the largest of the genera included in LAB. It is also very heterogeneous, encompassing species with a large variety of phenotypic, biochemical, and physiological properties.

Based on sugar fermentation patterns, LAB can be further divided into three broad metabolic categories (Axelsson, 1998). The first category includes the group I lactobacilli and some individual species from other genera that are obligately homofermentative, meaning the sugars can only be fermented by glycolysis. The second category includes leuconostocs, group III lactobacilli, oenococci, and weissellas that are obligately heterofermentative, meaning that only the 6-phosphogluconate/phosphoketolase (6-PG/PK) pathway is available for sugar fermentation. The apparent difference on the enzyme level between these two categories is the presence or absence of the key enzymes of glycolysis and the 6-PG/PK pathway. The third category, including the remaining LAB (i.e., group II lactobacilli and most species of enterococci, lactococci, pedicocci, streptococci, tetragenococci, and vagococci), holds an intermediate position. They resemble the obligately homofermentative LAB in that they possess a constitutive fructose-1,6-diphosphate aldolase, resulting in the use of glycolysis form hexose fermentation.

Other Bacteria In addition to LAB, other bacteria also are involved in food fermentations, some of which contribute significantly to flavor development and other characteristics of fermented products. Propionibacteria are probably best known for their role as dairy starter cultures, in which they produce the characteristic eyes and flavor of Swiss-type cheeses (Cogan and Accolas, 1996). The family Propionibacteriaceae, genus *Propionibacterium* and the closely related genus *Corynebacterium*, are classified as members of the Actinomycetaceae group. Five species of dairy propionibacteria are currently

Table 1. Differential characteristics of lactic acid bacteria at the genus level, based on morphology and physiology.

	Rods		Cocci						
Characteristic	*Carnobacterium*	*Lactobacillus*	*Enterococcus*	*Lactococcus Vagococcus*	*Leuconostoc Oenococcus*	*Pediococcus*	*Streptococcus*	*Tetragenococcus*	*Weissella*[a]
Tetrad formation	–	–	–	–	–	+	–	+	–
CO_2 from glucose[b]	–[c]	±	–	–	+	–	–	–	+
Growth:									
at 10°C	+	±	+	+	+	±	–	+	+
at 45°C	–	±	+	–	–	±	±	–	–
in 6.5% NaCl	ND[d]	±	+	–	±	±	–	+	±
in 18% NaCl	–	–	–	–	–	–	–	+	–
at pH 4.4	ND	±	+	±	±	+	–	–	±
at pH 9.6	–	–	+	–	–	–	–	+	–
Lactic acid[e]	L	D, L, DL	L	L	D	L, DL	L	L	D, DL

+, positive; –, negative; ±, response varies between species; ND, not determined.
[a]may also be rods.
[b]test for homo- or heterofermentation of glucose; +, homofermentation; –, heterofermentation.
[c]small amounts of CO_2 can be produced.
[d]no growth in 8% NaCl has been reported.
[e]configuration of lactic acid produced from glucose.
Modified from Axelsson (1998).

recognized: *P. freudenreichii* subsp. *freudenreichii*, *P. freudenreichii* subsp. *shermanii*, *P. thoenii*, *P. acidipropionici*, and *P. jensenii*. In addition to being important starter organisms in dairy fermentations, propionibacteria also contribute to natural fermentations of silage and olives and can produce a variety of industrially important products (Jay, 1996).

Acetobacters are Gram-negative aerobic rods and cocci and consist of three species, *Acetobacter aceti*, *A. pasteurianus*, and *A. peroxydans* (Lee, 1996). The organisms are widely distributed in nature where they are abundant in plant materials undergoing alcoholic fermentations. They are important for their role in the production of vinegar.

Fundamental Metabolism

The essential feature of LAB metabolism is efficient carbohydrate fermentation coupled to substrate-level phosphorylation. The generated adenosine triphosphate (ATP) is subsequently used for biosynthetic purposes. LAB as a group exhibit an enormous capacity to degrade different carbohydrates and related compounds. Generally, the predominant end product is lactic acid (>50% of sugar carbon). However, LAB adapt to various conditions and change their metabolism accordingly. This may lead to significantly different end-product patterns.

Carbohydrate Metabolism

LAB, as non-respiring microorganisms, principally generate ATP by fermentation of carbohydrates coupled to substrate-level phosphorylation. Many compounds can serve as fermentable growth substrates, and many pathways for their fermentation have evolved (Kandler, 1983). These pathways have the following three general stages (Thompson, 1988): 1) Conversion of the fermentable compound to the phosphate donor for substrate phosphorylation. This stage often contains metabolic reactions in which NAD^+ is reduced to NADH. 2) Phosphorylation of ADP by the energy-rich phosphate donor. 3) Metabolic steps that bring the products of the fermentation into chemical balance with the starting materials. The most frequent requirement in the last stage is a mechanism for oxidation of NADH, generated in the first stage of fermentation, to NAD^+ so that the fermentation can proceed. The two major pathways for metabolism of hexose in lactic acid bacteria are the homofermentative (Embden-Meyerhof) and heterofermentative (phosphoketolase) pathways (Figs. 1 and 2). The transport and phosphorylation of sugars occur according to the following metabolisms: transport of free sugar and phosphorylation by an ATP-dependent glucokinase for glucose; other sugars, such as mannose and fructose, enter the major pathways at the level of glucose-6-phosphate or fructose-6-phosphate after isomerization or phosphorylation or both (Axelsson, 1998). One exception is galactose metabolism in LAB which use the phosphoenolypyruvate:sugar phosphotransferase system (PTS) for uptake of this sugar. Some species of LAB use this system for all sugars, in which phosphoenolypyruvate is the phosphoryl donor.

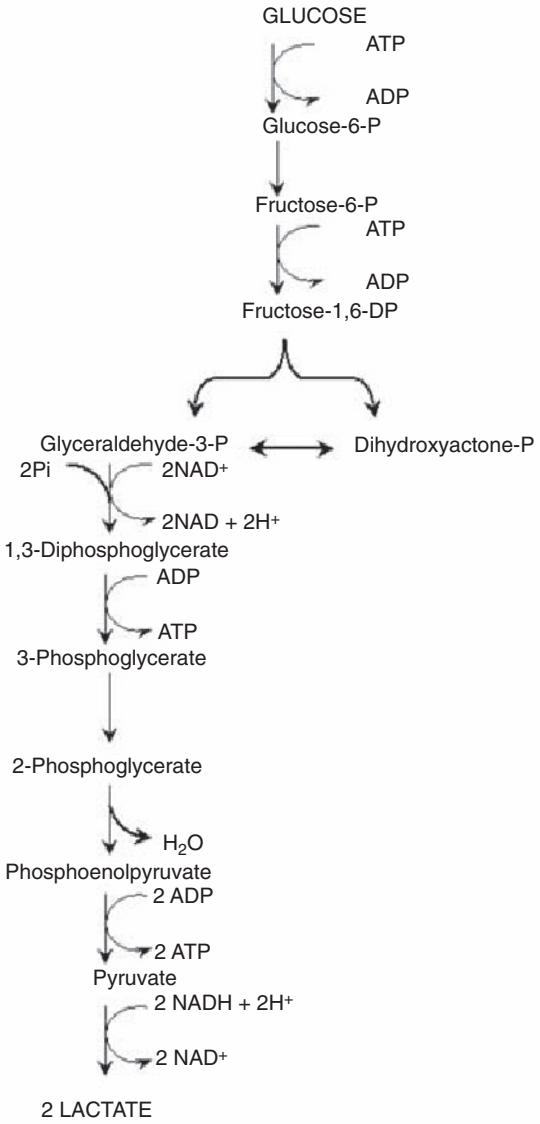

Fig. 1. Homolactic fermentation pathway of glucose (glycosis, Embden-Meyerhof pathway).

PROTEOLYSIS LAB have a very limited capacity to synthesize amino acids using inorganic nitrogen sources (Mayo, 1993). They are therefore

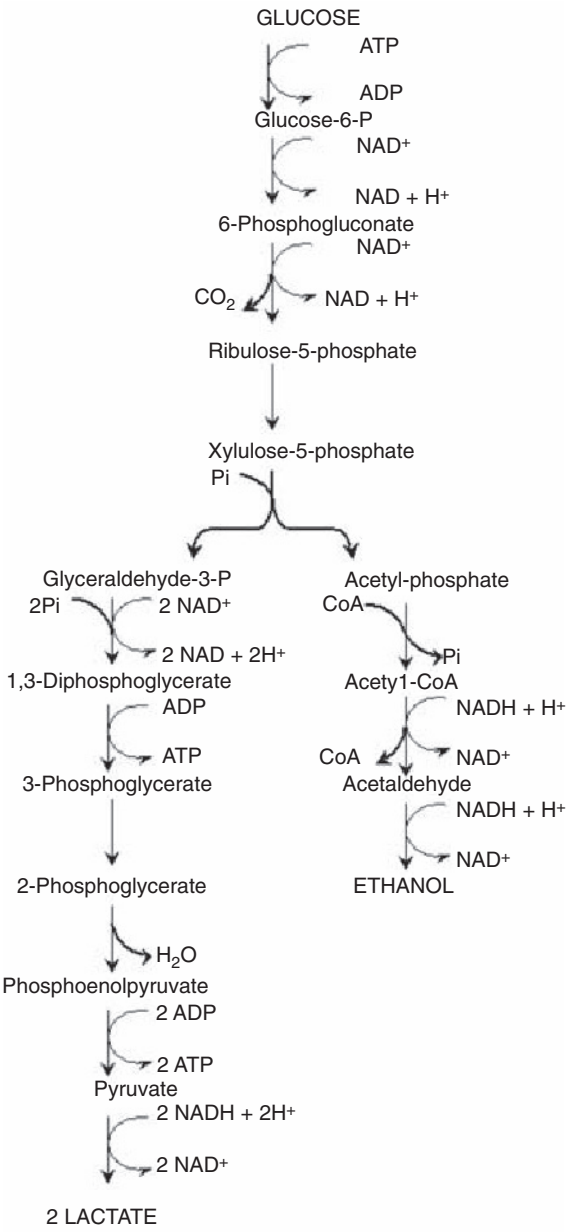

Fig. 2. Heterolactic fermentation pathway of glucose (6-phosphogluconate/phosphoketolase pathway).

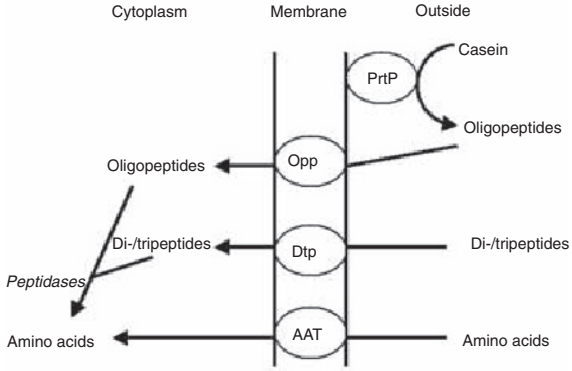

Fig. 3. Model of the lactococcal proteolytic system. Included also is transport of di- and tripeptides and free amino acids, but they contribute very little to the total growth of lactococci. PrtP, membrane-anchored proteinase; Opp, oligopeptide transport system; Dtp, di-/tripeptide transport system; AAT, amino acid transport system.

dependent on preformed amino acids being present in the growth medium as a source of nitrogen. The requirement for amino acids differs among species and strains within species (Chebbi et al., 1977). Some strains are prototrophic for most amino acids, whereas others may require 13–15 amino acids. The quantities of free amino acids present in food often are not sufficient to support the growth of bacteria to a high cell density; therefore, they require a proteolytic system capable of utilizing the peptides and proteins present in food that hydrolyzes proteins to obtain essential amino acids. All dairy lactococci used for acidification of milk (e.g., in cheese manufacture) have proteolytic activity (Poolman et al., 1995). The lactococcal proteolytic system consists of enzymes outside the cytoplasmic membrane, transport systems, and intracellular enzymes. The transport systems on the cell membrane include oligopeptide transport, di/tripeptide transport, and amino acid transport systems (Fig. 3; Pritchard and Coolbear, 1993). The transport of free amino acids contributes very little to the total growth of lactococci in milk. Two general classes of proteolytic enzymes of LAB are proteinases and peptidases. An extracellular, membrane-anchored serine proteinase (PrtP) has been identified as being essential for this activity. Once inside the cell, peptides are hydrolyzed by peptidases.

The proteolytic activity also contributes to the development of the flavor and rheological characteristics of fermented products (Mayo, 1993). For many varieties of cheeses, such as Swiss and Cheddar, desirable flavor tones are derived by proteolysis. However, proteolysis also can lead to undesirable flavors due to the accumulation of bitter peptides.

Bacteriocin Production Bacteriocins are protein antibiotics of relative high molecular weight that affect largely the same or closely related species by adsorption to receptors on the target cells. Bacteriocins can be added as a preservative (e.g., nisin), or they can be produced in the product (e.g., starter culture) or in the gastrointestinal tract (e.g., probiotic strains; De Vuyst and Vandamme, 1994; Montville and Winkowski, 1997). There are four classes of bacteriocins produced by LAB: 1) lantibiotics; 2) small hydrophobic

heat-stable peptides; 3) large heat-labile protein; and 4) complex bacteriocins (Table 2; Ouwehand, 1998).

Class I: Class I bacteriocins or lantibiotics are small peptides containing the unusual dehydroamino acids and thioether amino acids lanthionin and 3-methyllanthionine, which are synthesized by Gram-positive bacteria during posttranslational modifications. These peptides are thought to attach to the membrane of target cells and, by an as yet unknown conformational rearrangement, lead to increased permeability and disruption of the membrane potential. There are two types of lantibiotics, types A and B. The lantibiotics produced by LAB all belong to type A, which are elongated screw-shaped peptides, whereas type B lantibiotics are mainly globular. Nisin produced by *Lactococcus lactis* ssp. *lactis* has been studied extensively. It has a broad spectrum of activity against Gram-positive bacteria. The primary target is believed to be the cell membrane. Unlike some other antimicrobial peptides, nisin does not need a receptor for its interaction with the cell membrane; however, the presence of a membrane potential is required.

Class II: Class II bacteriocins contain a wide variety of small heat-stable peptides and have been subdivided into three subclasses (Table 2; Eijsink et al., 1998). Numerous class II bacteriocins are membrane-active peptides that destroy the integrity of the membrane by forming pores. Lactococcin is an example. In contrast to nisin, lactococcins require a specific receptor protein to act on target cells regardless of their membrane potential.

Classes III and IV: These bacteriocins differ markedly from those of Classes I and II. The larger (>30 kDa) heat-labile antimicrobial proteins such as lacacins A and B are classified as class III bacteriocins. Class IV bacteriocins such as leuconocin S and pediocin SJ-1 have lipid or carbohydrate moieties. The mechanisms of action and immunity of these complex bacteriocins remain unknown.

Starter Cultures

Starter cultures are food-grade microorganisms of known and stable metabolic activities and other characteristics that are used to produce fermented foods of desirable appearance, body, texture and flavor (Ray, 1996). Starter cultures in manufacture of food products were used long before it was known that bacteria were involved at all. Initial development of starter cultures resulted from the need and changes in the cheese industry. Since the early 1900s, there has been a marked worldwide increase in the industrial production of fermented food products. Process technology has progressed toward greater mechanization, larger factory size, shorter processing times, and more food processed daily in the processing plant. All of this relies on the optimization of the starter culture's activity, whereby the culture must maintain stable fermentative properties and resistance to bacteriophage (Lee, 1996). Currently, starter cultures for many types of fermented foods, including dairy products, meat products, vegetable products, and baking products, and for alcohol fermentation are commercially available.

Development of Starter Cultures Starter cultures used in dairy products can be divided into mesophilic and thermophilic cultures based on their optimum growth temperature (Table 3; Cogan and Accolas, 1996). Mesophilic cultures grow at temperatures of 10–40°C, with an optimum of ca. 30°C. Composed of acid-forming lactococci as well as flavor-producing bacteria, these cultures are used to make a variety of cheeses, fermented milk products, and ripened cream butter. Thermophilic cultures have optimum temperatures of 40–50°C and are used for yogurt and cheese varieties with high cooking temperatures. Starter cultures also have been used to produce meat products such as sausages and fermented vegetables. *Pediococcus acidilactici*, *Lactobacillus plantarum* and/or *Staphylococcus carnonsus* are often used as starter cultures

Table 2. Classes of bacteriocins produced by lactic acid bacteria.

Class	Subclass	Description	Bacteriocin producer
I		Lantibiotics	*Carnobacterium*, *Enterococcus*, *Leuconostoc*, *Lactobacillus*, *Streptococcus*
II		Small (<10kDa), heat stable (100–121°C)	*Carnobacterium*, *Enterococcus*, *Leuconostoc*, *Lactobacillus*, *Streptococcus*
	IIa	*Listeria*-active peptides	
	IIb	Two-peptide bacteriocins	
	IIc	Thiol-activated peptides	
III		Large (>30kDa) heat-labile proteins	*Lactobacillus*
IV		Complex bacteriocins: protein with lipid and/or carbohydrate	*Lactobacillus*, *Leuconostoc*, *Pediococcus*

Table 3. Examples of mesophilic and thermophilic starter culture organisms used by the dairy industry.

Type	Species
Mesophilic	*Lactococcus lactis* spp. *lactis*
	Lactococcus lactis spp. *cremoris*
	Lactococcus lactis spp. *lactis* var. *diacetylactis*
	Leuconostoc lactis
	Leuconostoc cremoris
Thermophilic	*Lactobacillus delbrueckii* spp. *lactis*
	Lactobacillus helveticus
	Lactobacillus delbrueckii spp. *bulgaricus*
	Lactobacillus acidophilus
	Streptococcus salivarius spp. *thermophilus*

in sausage production, whereas cabbage, cucumbers, and olives are fermented with *Lactobacillus plantarum*. Starter cultures, usually composed of different species or of several strains of a single species, are often categorized on the basis of their composition (Ray, 1996): 1) single-strain starter: one strain of a certain species; 2) multiple-strain starter (defined-strain starter): different known strains of one species; 3) multiple-mixed-strain starter: different defined strains of different species; and 4) raw mixed-strain starter: species and strains partly or all unknown.

General Requirements for Starter Cultures (Buckenhuskes, 1993)

Safety Starter cultures contain bacteria that lack virulence factors for humans and thus are free of pathogens and toxins.

Technological Effectiveness Starter culture bacteria dominate over naturally occurring microflora. The microorganisms perform the required metabolic activities. Starter culture preparations are free of bacteriophage and microorganisms that may inhibit or reduce starter culture activity.

Economic Aspects The propagation and production of starter cultures must be economically feasible. The starter culture can be preserved by freezing or freeze-drying with little practical loss of activity. Essential properties of starter cultures such as fermentative ability are stable under defined storage conditions for several months.

Frozen concentrated starter cultures were developed in the 1970s for direct inoculation into substrates to be cultured. Efforts have been made to produce freeze-dried concentrated cultures, which have less bulk and don't require transport on dry ice, thereby eliminating the problem of accidental thawing. The dried cultures can be used directly for product manufacture or to produce bulk starters (Cogan and Accolas, 1996). However, many starter culture strains do not survive well in the dried state. Hence, the care of dried cultures in large commercial operations has been limited. A recent advance has been the availability of custom-designed starter cultures to meet the specific needs of a food processor. An understanding of the genetic basis of both desirable fermentative characteristics as well as phage inhibition defenses of starter cultures has advanced the production of designer cultures.

Genetics of LAB Starter Cultures

The characterized genetic elements of LAB starter cultures include chromosomes, transposable elements, and plasmids (Gasson, 1990; Rodriguez and Vidal, 1990). Chromosomes of LAB are smaller than those of other eubacteria, ranging from 1.1 to 2.6 Mbp, depending on the species. Transposable elements, genetic elements capable of moving as discrete units from one site to another in the genome, have been identified in LAB. Insertion sequences, the simplest of transposable elements, are widely distributed in bacteria and also have been found in LAB. Their ability to mediate molecular rearrangements and affect gene regulation has had both positive and negative implications for food fermentations (Gasson, 1990). Plasmids have been identified in many LAB. Some plasmids encode many of the activities essential for food fermentations (de Vos, 1999), including lactose metabolism, proteinase activity, oligopeptide transport, bacteriophage resistance mechanisms, bacteriocin production and immunity, bacteriocin resistance, exopolysaccharide production, and citrate utilization.

Natural gene transfer systems among LAB are largely transduction and conjugation, although transformation also has been reported as a means of genetic exchanges (Rodriguez and Vidal, 1990). Transduction, the transfer of bacterial genetic material by a bacteriophage, has been demonstrated in lactococci, lactobacilli, and *Streptococcus thermophilus*. Usefulness of transduction in construction of strains for the fermentation industry is limited because of the relatively narrow host range of transducing bacteriophage. Conjugation, the transfer of genetic material from one bacterial cell to another, which requires cell-to-cell contact, has been well characterized in lactococci. Most plasmid-encoded characteristics important in the manufacture of fermented dairy products can be transferred by conjugation. Using an approach that does not require antibiotic-resistance markers, conjugation has been used to transfer plasmids that encode bacteriophage-resistance genes into commercial lactococcal strains (Verrips and van den Berg, 1996). These strains have enhanced resistance to infection by bacterioph-

age and have been used successfully in the dairy industry for years.

Starter Culture Improvement Past emphasis on starter culture improvement was largely based on screening natural isolates for traits of interest and monitoring existing strains to select for beneficial variations. More recently, advances in molecular technology have enabled us to understand more about the biology, physiology, and taxonomy of LAB and other microorganisms important to food fermentations (McKay and Baldwin, 1990). In the future, it may be possible to definitively identify and then combine the most desirable nutritional, sensory, and/or therapeutic properties of starter cultures to construct "superior" strains for food fermentations (Geisen and Holzapfel, 1996). At present, however, strain improvement focuses on eliminating problems that beset their use as starter cultures. Features such as bacteriophage infection, genetic instability, variation and unpredictability in performance, and the production of low-grade or poor-quality products all lead to economic losses. These are characteristics that have been examined extensively with interest in identifying and generating strains with superior attributes. Considerable research has been devoted to studying phage and phage-host interactions in LAB, the mechanisms by which lactose, citrate, and protein are metabolized, and the basis for instability and unpredictability in strain performance. Also because of their food-grade, nonpathogenic, generally recognized as safe (GRAS) status, LAB are considered to be ideal hosts for the production of proteins and other compounds that they do not produce naturally and that have medical or food-related applications (Kuipers et al., 1997).

Bacteriophage and Bacteriophage Resistance Food fermentations rely on actively growing LAB that either are added as starter cultures or grow spontaneously in the food matrix. The fermentation capabilities of LAB can be severely inhibited by bacteriophage infection, which has been a major commercial problem (Klaenhammer, 1991). Bacteriophages are bacterial viruses that were first identified as "filter-transmissible" agents at the beginning of 1900. Significant progress has been made toward the characterization of bacteriophages from LAB. All of the bacteriophages examined contain double-stranded linear DNA genomes with either cohesive or circularly permuted terminally redundant ends. Both lytic and temperate bacteriophages have been identified. Bacteriophage infection may lead to a decrease or complete inhibition of lactic acid production by the starter culture. This effect has had a major impact on the manufacture of fermented food products, as lactic acid synthesis is required to produce these products. In addition, slow acid production disrupts manufacturing schedules and typically results in products that are of lower economic value. Therefore, bacteriophage resistance remains one of the most important characteristics of any industrial LAB strain, whether natural or genetically engineered (Klaenhammer, 1991).

Selective environmental pressure placed on LAB by bacteriophages over thousands of years has resulted in strains that possess many bacteriophage defense mechanisms. The best-characterized bacteriophage-resistant LAB is lactococci (Dinsmore and Klaenhammer, 1995; Sanders, 1988). Phage defense mechanisms identified in lactococci include abortive infection mechanisms, the restriction-modification system, and interference with phage adsorption. Abortive phage infection is a powerful defense, acting after phage injection to decrease phage development efficiency and so reduce the number of emerging phages when the cell bursts. This mechanism effectively decreases phage replication rates and results in poor to no plaque formation on agar assays. The restriction modification system is another common phage defense mechanism that reduces plaquing efficiencies of phages on normally permissive hosts by several log cycles. Such systems operate by coordinated activities of a restriction enzyme that recognizes and cleaves foreign DNA and a modification enzyme that labels DNA as host derived. These defense loci are encoded by plasmids capable of conjugal transfer, suggesting that genetic exchange between LAB has an important role in the development of bacteriophage-resistant starter cultures (Sanders, 1988). Recombinant DNA techniques also have been used to engineer starter cultures with enhanced bacteriophage resistance (Daly et al., 1996). An alternative approach to the development of phage-resistant strains uses a designed antisense RNA to control the expression of phage genes.

Flavor Production LAB generate a range of flavor products such as diacetyl, which has a buttery aroma and is a highly desirable product in many foods (Cogan and Accolas, 1996). Strategies involving metabolic engineering, whereby metabolic pathways are manipulated to overproduce specific products, have been used to develop cultures that elaborate elevated levels of diacetyl. Proteolytic activity of LAB is of major significance in contributing to the liberation of small peptides and amino acids which either add flavor directly or are likely to be flavor precursors.

Commercial Application

DAIRY PRODUCTS Fermented dairy products are enjoying increased popularity as convenient, nutritious, stable, natural, and healthy foods. LAB are the principal organisms involved in the manufacture of cheese, yogurt, sour cream, and cultured butter. In some fermented dairy products, additional bacteria, referred to as secondary microflora, are added to produce carbon dioxide, which influences the flavor and alters the texture of the final product (Early, 1998). Two LAB, *Leuconostoc* species and strains of *Lactobacillus lactis* subsp *lactis*, which are capable of metabolizing citric acid, are added to produce aroma compounds and carbon dioxide in cultured buttermilk and certain cheeses. *Propionibacterium freudenreichii* subsp. *shermanii* is added to Swiss-type cheeses primarily to metabolize L-lactic acid to propionic acid, acetic acid, and carbon dioxide. Carbon dioxide forms the eyes in Swiss-type cheeses. Other types of secondary microflora include undefined mixtures of yeasts, molds, and bacteria (Ray, 1996). These microorganisms are added directly to the milk or are smeared, sprayed, or rubbed onto the cheese surface. This group of microorganisms has extremely varied and complex metabolic activities, their main function being to produce unique flavors. LAB and the predominant microbes used to make fermented dairy products are listed in Table 4 (Johnson and Steele, 1997).

Cultured Butter and Buttermilk Cultured butter is made from milk fat to which a mesophilic starter culture has been added to enhance its flavor, principally that of diacetyl. Diacetyl, made from citrate by LAB, enhances buttermilk's storage properties. *Lactobacillus lactis* or mixed cultures that contain *Lb. lactis*, *Leuconostoc citrovurum*, and *Leu. dextranicum* are used (Early, 1998). Fat (cream) is separated from skim milk by centrifugation of milk. The cream is pasteurized and inoculated with selected starter cultures. The ripened cream is then churned. The cream separates again into cream butter and its byproduct sour buttermilk, which has limited use because of its high acidity.

An alternative process has been developed to produce cultured butter without the formation of sour buttermilk. In this process lactose-reduced whey inoculated with *Lactobacillus helveticus*, and skim milk inoculated with a starter culture to produce aroma compounds and lactic acid, are added to the pasteurized cream. The cream is further churned and worked. The resulting butter is known as sour aromatic butter and the liquid phase is sweet buttermilk, which is not as acidic as sour buttermilk.

Yogurt Yogurt is produced by fermenting milk, usually cow's milk, with *Streptococcus thermophilus* and *Lactobacillus bulgaricus* (Chandan and Shahani, 1993). Initial acid production is largely due to *Str. thermophilus*, but the characteristic yogurt flavor is produced by *Lb. bulgaricus*. Both yogurt cultures may produce extracellular polymers, which contribute to the viscosity of yogurt. It is desirable that the starter

Table 4. Microorganisms involved in the manufacture of fermented dairy products.

Product	Principal acid producers	Secondary microflora
Cheese		
Colby, Cheddar, cottage, cream	*Lactococcus lactis* subsp. *cremoris* *Lactococcus lactis* subsp. *lactis*	None
Blue	*Lactococcus lactis* subsp. *cremoris* *Lactococcus lactis* subsp. *lactis*	Cit$^+$ *Lactococcus lactis* subsp. *lactis* *Penicillium roqueforti*
Mozzarella, provolone, Romano, parmesan	*Streptococcus thermophilus* *Lactobacillus delbrueckii* subsp. *bulgaricus* *Lactobacillus helveticus*	None
Swiss	*Streptococcus thermophilus* *Lactobacillus delbrueckii* subsp. *bulgaricus* *Lactobacillus helveticus*	*Propionibacterium freudenreichii* subsp. *shermanii*
Fermented milk		
Yogurt	*Streptococcus thermophilus* *Lactobacillus delbrueckii* subsp. *bulgaricus*	None
Buttermilk	*Lactococcus lactis* subsp. *cremoris* *Lactococcus lactis* subsp. *lactis*	*Leuconostoc* sp. Cit$^+$ *Lactococcus lactis* subsp. *lactis*
Sour cream	*Lactococcus lactis* subsp. *cremoris* *Lactococcus lactis* subsp. *lactis*	None

cultures be present in approximately equal numbers to ensure the characteristic flavor, consistency and odor; otherwise *Lb. bulgaricus* becomes dominant.

Yogurt is generally fermented in batches. Standardized milk, with a fat content of 0.5 to 3.0% and milk solids of 14 to 16%, is homogenized and pasteurized. After cooling to the incubation temperature, the batch is inoculated with starter cultures and incubated for 16 h at 30°C or 4 h at 45°C. The product is then cooled to 2 to 4°C and packaged and stored.

Cheese Cheese making is essentially a dehydration process in which milk casein, fat, and minerals are concentrated 6 to 12-fold, depending on the variety. Although the manufacturing protocols for individual varieties differ, the basic steps common to most varieties are acidification, coagulation, dehydration, and salting. Acid production is the major function of the starter bacteria. Lactic acid is responsible for the fresh acidic flavor of unripened cheese and is of importance in coagulation of milk. Coagulation of the casein is accomplished by the concerted action of rennet and by acidification. Starters play other essential roles in the production of volatile flavor compounds (e.g., diacetyl, aldehydes), the synthesis and release of the intracellular proteolytic and lipolytic enzymes involved in the cheese ripening (Steele, 1995), and the suppression of pathogens and other spoilage microorganisms (Lewus et al., 1991). For cheddar cheese production, starters include mixed strains of *Lactobacillus lactis* subsp. *cremoris* or *lactis*. *Leuconostoc* may be added for flavor development. *Streptococcus thermophilus* and *Lb. helveticus* are used in Swiss cheese primarily for acid production, whereas *Propionibacterium* spp. is a secondary culture added for eye formation, taste, and flavor. Similar to Cheddar cheese production, blue cheese production requires *Lb. lactis* subsp. *cremoris* or *lactis* and *Leuconostoc* spp, but the mold *Penicillium roqueforti* is also added as a secondary culture for flavor and blue appearance.

Meat Products Fermented meat products are defined as meats that are deliberately inoculated during processing to ensure sufficient controlled microbial activity to alter the product's sensory characteristics (Luche, 1994). Dry and semidry sausages represent the largest category of fermented meat products, with many present-day processing practices having their origin in the Mediterranean region. Fermented sausages are chopped or ground meat products that, as a result of bacterial action, reach a pH of 5.3 or less and are then dried. Based on final moisture content, fermented sausages are classified as dry (25–45% moisture content) or semidry (40–50% moisture content). To ensure products of consistent flavor, texture and shelf stability, as well as to improve product safety, most processors have developed pure microbial cultures to control the fermentation of their sausage product. It is evident that with a starter culture, the pH drops much more rapidly, hence the entire manufacturing process is accelerated, leading to economic benefits for the processor. Most starter cultures are natural isolates of the desirable microorganisms found normally in sausage (Egan, 1983). A wide variety of species has been used as starter cultures in meat and poultry products (Table 5; Riche and Keeton, 1997).

Table 5. Bacteria used as starter cultures in meat and poultry products.

Products	Bacteria
Semi-dry fermented meat sausages	
Lebanon bologna	Mixture of *Pediococcus cerevisiae*/*actobacillus plantarum*
Summer sausage	*P. cerevisiae* or mixture/*Lb. plantarum*
Cerevelat	*P. cerevisiae* or mixture/*Lb. plantarum*
Thuringer	*P. cerevisiae*
Teewurst	*Lactobacillus* species
Pork roll	*P. cerevisiae*
Dry fermented meat sausages	
Pepperoni	*P. cerevisiae*/*Lb. plantarum*
Dry sausage	*P. cerevisiae*
European dry sausage	*Micrococcus* spp. or *Micrococcus*/*Lactobacillus* spp.
Salami	*Micrococcus*/*Lactobacillus* spp. or *Lb. plantarum*
Hard salami, Genoa	*Micrococcus* spp.
	Micrococcus spp./*P. cerevisiae; Micrococcus* spp./*Lb. plantarum*
Fermented poultry sausages	
Semi-dry turkey sausage	*P. cerevisiae*
Dry turkey sausage	*P. cerevisiae*/*Lb. plantarum*

VEGETABLE AND FRUIT PRODUCTS The fermentation of vegetables and fruits can be affected by many different microorganisms (Daeschel and Fleming, 1984). LAB and yeasts are preferentially used in the western hemisphere, whereas in the Orient a large number of victuals are fermented by molds. However, the most extensively used procedure for biopreservation of vegetables involves lactic acid fermentation. Almost all vegetables can be fermented through natural processes because they contain fermentable carbohydrates and harbor many types of LAB. Therefore, many fermented vegetable products are produced by natural fermentation, and some such as cucumbers are now being produced by controlled fermentation (Buckenhuskes, 1997). Although many different vegetables are commercially fermented, at present only olives, cabbage for sauerkraut, and cucumbers for pickles are of major economic importance (Buckenhuskes, 1993).

Sauerkraut Sauerkraut is produced from the natural lactic acid fermentation of cabbage that has been shredded and salted. It is a major fermented vegetable food in Europe and the United States. At the beginning of the fermentation, some oxygen remains in the shredded cabbage. Plant cells, aerobic bacteria, yeasts and molds consume this remaining oxygen and then die off as the oxygen supply diminishes. The facultative anaerobes then increase in number. Initially, coliform species (e.g., *Enterobacter cloacae*) and *Flavobacterium* species grow to produce gas, volatile fatty acids and flavors. As the acidity increases, these bacteria are normally replaced by *Leuconostoc mesenteroides*, which become the predominant microbes. They are subsequently succeeded by *Lactobacillus brevis*, *Pediococcus pentosaccus* and *Lb. Plantarum*, which raise the acid level to about 2% and decrease the pH to 3.4–3.6.

Cucumber Pickles The starter culture for cucumber fermentation usually consists of the normal mixed flora of cucumbers, including *Lb. mesenteroides*, *Enterococcus faecalis*, *Pediococcus cerevisiae*, *Lb. brevis*, and *Lb. plantarum*. Of these, the pediococci and *Lb. plantarum* are the most involved, with *Lb. brevis* being undesirable because of its ability to produce gas. *Lb. plantarum* is the most essential species in pickle production, as it is for sauerkraut. The natural fermentation of cucumbers, though in practice for many years, can lead to pickle spoilage and thereby serious economic loss. A controlled fermentation of cucumbers brined in bulk has been developed, and this process not only reduces economic losses but also leads to a more uniform product over a shorter period of time (10–12 days). This method employs a chlorinated brine of 25° salinometer, acidification with acetic acid, the addition of sodium acetate, and inoculation with *P. cerevisiae* and *Lb. plantarum* or with *Lb. plantarum* only.

Olives Like cucumbers, olives are fruits that are categorized as vegetables and are fermented under conditions similar to those of other vegetable products. The microbial population responsible for the fermentation of olives differs from that of sauerkraut and pickles mainly because the higher salt concentration of the brine prevents many salt-sensitive strains from growing and provides an advantage to salt-tolerant strains. LAB become prominent during the intermediate stage of fermentation. *Lb. mesenteroides* and *P. cerevisiae* are the first lactics to become predominant, followed by lactobacilli, with *Lb. plantarum* and *Lb. brevis* being the most important. The fermentation may require as long as 6–10 months to complete, and the final pH of the product is 3.8 to 4.0.

Vinegar Vinegar (acetic acid) is used as an acidulant and flavor compound in processed foods. It is produced by an alcoholic fermentation of sugar-containing plant extracts followed by a microbial oxidation of ethanol to acetic acid (Sievers and Teuber, 1995). The slow process of natural acetification of wines and ciders is the oldest method of making vinegar. The Orleans process, developed in France for the industrial production of vinegar at the end of the 14th century, is also a slow fermentation in which the substrate "wine" is placed in barrels and inoculated with fresh vinegar or with the slimy, thick microbial film formed on the surface of the fermenting mash during acetification. Technological advances have led to a submerged culture process in which a suspension of *Acetobacter* spp. grows in an ethanol-containing substrate with constant agitation and small air bubbles. Commercial strains of *Acetobacter* include low cost, higher yields, less space required and low evaporation loss.

Malolactic Fermentation in Wine Malolactic fermentation (carried out by many LAB) involves the decarboxylation of malic acid to lactic acid and CO_2. In wine making as well as in production of cider and perry, LAB reduce acidity by converting malic to lactic acid and modify flavor and texture, which in part mature a beverage (Henick-Kling, 1995). Red and white wines are commonly produced by a yeast alcoholic fermentation of musts prepared from grapes. After alcoholic fermentation, wines frequently undergo malolactic fermentation. This process occurs naturally at or near the completion of alcoholic

fermentation. Wines produced from grapes cultivated in cool climates generally have higher concentrations of malic acid, which can mask the varietal character of the wine. Decreasing acidity by malolactic fermentation produces a wine with a softer and more mellow taste. LAB resident in wine are responsible for the malolactic fermentation. However, since the early 1980s, commercial starter cultures consisting of *Leuconostoc oenos*, *Lb. plantarum*, and *Lb. hilgardii* as single- or multiple-strain preparations have been available for the induction of malolactic fermentation (Buckenhuskes, 1993). The malolactic fermentation is more commonly used in red wines, although recently it is increasingly used in white wines.

Indigenous Fermented Beverages Production of indigenous fermented beverages (examples listed in Table 6) often involves complex biochemical, sensory and nutritional changes that can result from more or less controlled microbial activity in a range of raw materials (Beuchat, 1997). The preparation of many indigenous or traditional fermented beverages is a household art. Thus, they are often produced regionally or by different ethnic groups and are not commerically available (Steinkraus, 1983). The microbiology of indigenous fermented beverages is less clear. In many cases, LAB and yeasts both contribute to the fermentation process.

Probiotic Bacteria and Competitive Exclusion

A probiotic is a mono- or mixed culture of live microorganisms that, applied to animals or humans, beneficially affects the host by improving the properties of the gastrointestinal normal flora (Abe et al., 1995; Holzapfel et al., 1998; Hove et al., 1999). But it is restricted to products that contain live organisms, improve the health and well-being of animals or humans, and can have their effect on all host mucosal surfaces. Most microbial species used or tested for probiotic efficacy are LAB. Some bifidobacteria and yeasts are also used in the dairy industry. LAB have been used in foods for centuries and most strains are considered commensal microorganisms with no pathogenic potential. Their omnipresence on the intestinal epithelium of the human gastrointestinal tract, their traditional use in fermented foods without significant health-associated problems, and their health-promoting benefits make this group of bacteria ideal candidates for use as a probiotic (Naidu et al., 1999).

Probiotic Bacteria in Food Incorporating probiotic bacteria into foods to counteract harmful bacteria in the intestinal tract has been a renewed interest in health promotion and disease prevention in recent years (Gilliland, 1990; Gorbach, 1990; Holzapfel et al., 1998; Hove et al., 1999). Foods containing probiotic microorganisms range from dairy foods to infant formulas, baby foods, fruit juice-based products, cereal-based products, and pharmaceuticals. New and more specific strains of probiotic bacteria are being sought. Many studies have been conducted during the last 30 years to determine specific health benefits from the consumption of live cells of these organisms (Gorbach, 1990; Hove et al., 1999). There are three major sources of the live cells: 1) fermented milk products, such as yogurt containing live cells of *Lb. delbrueckii* subsp. *bulgaricus* and *Str. thermophilus* and acidophilus milk containing *Lb. acidophilus*; 2) supplementation of foods and drinks with live cells of one or more types of probiotic bacteria, such as *Lb. acidophilus*, *Lb. reuteri*, *Lb. casei*, and *Bifidobacterium* species; and 3) pharmaceutical products in the form of tablets, capsules, and granules. The beneficial effects of probiotic bacteria include protection against enteric pathogens (Jeppesen and Huss, 1993; Lewus et al.,

Table 6. Examples of indigenous fermented beverages.

Product	Geography	Substrate	Microorganism(s)	Product use
Busa	Tartars of Krim, Turkestan, Egypt	Rice or millet, sugar	*Lactobacillus* and *Saccharomyces* spp.	Drink
Mahewu (Magou)	South Africa	Maize	LAB	Sour and nonalcoholic drink
Pito	Nigeria	Guinea corn or maize or both	Yeast, LAB	Drink
Sorghum beer	South Africa	Sorghum, maize	LAB, yeast	Acidic and weakly alcoholic drink
Soybean milk yogurt	China, Japan	Soybeans	LAB	Drink
Pulque	Mexico	Agave	LAB, *Saccharomyces* spp., *Zymomonas mobilis*	Alcoholic drink

LAB, lactic acid bacteria.

1991; Okereke and Montville, 1991; Rodriguez et al., 1997), detoxification and improved digestion by means of enzymes to metabolize some food nutrients (e.g., lactase to hydrolyze lactose; Gilliland, 1990) and remove some harmful food components and metabolites (El-Nezami and Ahokas, 1998), stimulation of the intestinal immune system (Salminen and Deighton, 1992; Salminen and Salminen, 1997), and improvement of intestinal peristaltic activity (Rafter, 1995).

Selection Criteria for Probiotic Bacteria The theoretical base for selection of probiotic microorganisms includes safety, functional aspects (survival, adherence, colonization, antimicrobial production, immune stimulation, antigenotoxic activity, and prevention of pathogens), and technological aspects (growth in milk or other food base, sensory properties, stability, phage resistance, and viability; Salminen et al., 1998).

Competitive Exclusion Concept The competitive exclusion concept was originated by Nurmi and Rantala (1973), based on the study of *Salmonella* in chickens. Newly hatched birds on modern poultry farms are not able to obtain the normal gut flora of adult birds. As a result, the intestines of chicks are easily colonized by pathogens. When the chickens were inoculated immediately after birth with the intestinal content of a *Salmonella*-free adult bird, the frequency of Salmonella infections was radically reduced and the number of *Salmonella* needed to colonize the ceca of chicks increased. Hence, the normal gastrointestinal microflora of adult chickens can competitively exclude *Salmonella* from colonizing the naïve intestinal tract of chicks.

Competitive Exclusion of Foodborne Pathogens After establishment of the basic concept of competitive exclusion, considerable research has been done to identify the mechanisms by which bacteria are competitively excluded from host sites (Nurmi et al., 1992; Zhao et al., 1995). Responsible factors include competition for receptor sites on the intestinal epithelium, production of volatile fatty acids and/or other antibacterial substances, and competition among different bacteria for limited nutrients. The use of LAB as a probiotic for live poultry and livestock has been extensively studied and has potential to reduce carriage of pathogens and increase growth rates (Abe et al., 1995; Hammes and Tichaczek, 1994). Cecum-colonizing bacteria including *Escherichia coli* that produce inhibitory metabolites to *Campylobacter jejuni* have been used successfully to reduce intestinal carriage of *C. jejuni* by poultry (Aho et al., 1992; Schoeni and Doyle, 1992). Similarly, *E. coli* strains that produce antimicrobial metabolites to *E. coli* O157:H7 have been confirmed effective in reducing or eliminating carriage of *E. coli* O157:H7 by cattle (Zhao et al., 1998).

Prospects

For many years bacteria, mainly LAB, have been involved in the fermentation of foods from raw agricultural materials such as milk, meat, vegetables, fruits, and cereals. Fermented foods are a significant part of the food processing industry and are often produced using bacteria that have been selected for their ability to effectively produce desired products or changes. The interaction of LAB and other bacteria in enhancing the physiology, nutrition, and metabolism of humans and animals, and their involvement in promoting health and reducing disease, are additional roles that prokaryotes can serve by their presence in foods. Over the past decade, there have been major developments in furthering our understanding of both the biochemistry and physiology of bacteria involved in food fermentations. Advanced molecular techniques have served as invaluable tools for the development of defined mutants that have enabled basic studies on proteolysis, peptidase action, and peptide transport. Knowledge from such studies is invaluable for the design and modification of commercially useful strains. The application of genetic engineering technology to improve existing strains through enhanced bacteriophage resistance or more efficient metabolic characteristics such as proteolytic activities or to develop novel strains for fermentations has greatly contributed to the success of the fermentation industry through more consistent production of high quality, uniform products and less fermentation failure.

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