CHAPTER 2

LITERATURE REVIEW

2.1 Lactic Acid Bacteria

2.1.1 General Introduction

Lactic acid bacteria (LAB) are a group of Gram-positive bacteria united by a constellation of morphological, metabolic and physiological characteristics. The general description of the bacteria included in the group is Gram-positive, non-sporing, non-respiring cocci or rods, which produce lactic acid bacteria as the major end product during the fermentation of carbohydrates. LAB are generally associated with habitats rich in nutrients, such as various food products (milk, meat, vegetables), but some are also members of the normal flora of the mouths, and vagina of mammals. Variations of this genera theme are common, excluding the Gram-positive and nonsporing characters, which cannot be disputed (spore-forming bacteria that resemble LAB, e.g., *Sporolactobacillus*, are more related to bacilli) (Salminen and Wright, 1993).

Two main sugar fermentation pathways can be distinguished among lactic acid bacteria. Glycolysis (Embden-Meyerhof pathway) results in almost exclusively lactic acid as end product under standard condition and the metabolism is referred to as homolactic fermentation. The 6-phosphogluconate or phosphoketolase pathway results in significant amounts of other end products, such as ethanol, acetate and CO_2 in addition to lactic acid and the metabolism is referred to as heterolactic fermentation (Salminen and Wright, 1993). The general metabolism and physiology of LAB reflects their adaptation niches rich in nutrients. They have developed very efficient transport systems, which enable them to quickly take up the necessary solutes. The extreme sacharolytic nature is another example. Very few LAB can use compounds other than carbohydrates (and related substances such as mannitol, etc.) as sole energy sources. However, as tried to emphasize, they have developed systems that will allow them to derive more energy from a rich medium than just a carbohydrate. One of these systems are the co-fermentations that have been mentioned several times. By using a substrate, otherwise, as an electron acceptor during carbohydrate fermentation, they indirectly derive some energy from that substrate that otherwise would be lost. The malo-lactic fermentation serves the same purpose, whatever the actual mechanism for energy transduction may be. The use of co-fermentations may also enable LAB to extend the range of fermentable substrates. For example, *Lb. plantarum* is unable to ferment either citrate or mannitol anaerobically. However, added together, both substrates are used, mannitol as the main energy sources and citrate as electron acceptor.

Lactic acid bacteria are widespread in nature in soil, vegetables, meat, milk and the human body. Many are used in fermented dairy products. *Sterptococcus thermophilus* (*S. thermophilus*) and *Lactobacillus bulgaricus* (*L. bugaricus*) are the two bacteria required to make yoghurt. Many commercial yoghurts in Canada also contain *Lactobacillus acidophilus* (*L. acidophilus*) and *Bifidobacterium bifidum* (*B. Bifidum*). *Lactobacillus casei* (*L. casei*) is frequently found in cheeses. All these species of bacteria are believed to be probiotic. These species can be further classified into subspecies, variants and strains. The difficulty in identifying and classifying strains has complicated research, since benefits may only pertain to particular strains.

Lactic Acid Bacteria were first isolated from milk (Carr *et al*., 2002) and have since been found in such foods and fermented products as meat, milk products, vegetables, beverages and bakery products (Aukrust and Blom, 1992). Lactic Acid Bacteria occur naturally in fermented food and have been detected in soil, water, manure and sewage (Holzapfel *et al*., 2001). Lactic Acid Bacteria exist in human and in animal (Fuller and Brooker, 1974) , however some lactic acid bacteria are part of the oral flora which can cause dental caries. They can work as spoilage organisms in foods such as meat, fish and beverages (Liu, 2003). Lactic acid Bacteria have been used as a flavoring and texturizing agents as well as a preservative in food for centuries and are now added as starters in food such as *Lactobacilli*, *L. lactis* and *Streptococcus thermophilus* , inhibit food spoilage and pathogenic bacteria and preserve the nutritive qualities of raw food material for an extended shelf life (Heller, 2001)

2.1.2 Classification at the Genus Level

The classification of lactic acid bacteria into different genera is largely based on morphology, mode of glucose fermentation, growth at different temperatures, configuration of the lactic acid produced, ability to grow at high salt concentrations and acid or alkaline tolerance. For some of the newly described genera, additional characteristics such as fatty acid composition and motility are used in classification. The measurements of true phylogenetic relationships with rRNA sequencing have aided the classification of lactic acid bacteria (LAB) and clarifird the phylogeny of group. Most genera in the group form phylogenetically distinct group, but some, in particular *Lactobacillus* and *Leuconostoc* are very heterogeneous and thee phylogenetic clusters do not correlate with the current classification based on phenotypic characters. New tools for classification and identification of lactic acid bacteria are underway. The most promising for routine use are nucleic acid probing techniques, partial rRNA gene sequencing using the polymerase chain reaction and soluble protein patterns.

The genera that, in most respects, fit the general description of the typical LAB are (as they appear in the latest edition of *Bergey's Manual* from 1986) *Aerococcus* (*A.*), *Lactobacillus* (*Lb.*), *Leuconostoc* (*Ln.*), *Pediococcus* (*P.*), and *Streptococcus* (*S.*). Majors revisions of the taxonomy of LAB, in particular of the streptococci, was anticipated in *Bergey's Manual* from 1986 (Hardie, 1986b; Schleifer, 1986) and to some extent already realized by the year of that issue. Thus, the former genus *Streptococcus* was first divided into three: *Enterococcus* (*E.*), *Lactococcus* (*Lc.*), and *Streptococcus sensu stricto* (Schleifer and Kilpper-Balz, 1987; Schleifer *et al*, 1985). Later, some motile LAB, otherwise resembling lactococci, were suggested to form a separate genus, *Vagococcus* (*V.*) (Collins *et al.*, 1989). The genera

Lactobacillus and *Pediococcus* have largely remained unchanged, but some rod-shaped LAB, previously included in *Lactobacillus*, are now forming the genus *Carnobacterium* (*C.*) (Collins *et al.*, 1987), and the former species *Pediococcus halophilus* has been raised to genus level, forming the genus *Tetragenococcus* (*T.*) (Collins *et al.*, 1990). Revisions after 1986 are supported by extensive chemotaxonomic and genetic data. Further revisions are to be expected with regard to the genera *Lactobacillus, Leuconostoc* and *Pediocccus*.

Although morphology is regarded as questionable as a key character in bacterial taxonomy (Woese, 1987). It is still very important in the current descriptions of the LAB genera. Thus, LAB can be divided into rod (*Lactobacillus* and *Carnobacterium*) and cocci (all other genera). Furthermore, cell division in two planes, leading to tetrad formation, is used as a key characteristic in the differentiation of the cocci. The tetrad-forming are *Aerococcus*, *Pediococcus* and *Tetragenococcus*. An important characteristic used in the differentiation of the LAB genera is the mode of glucose fermentation under standard conditions. i.e., nonlimiting concentrations of glucose and growth factors (amino acids, vitamins and nucleic acid precursors) and limited oxygen availability. Under these conditions, LAB can be divided into two groups: homofermentative, which convert glucose almost quantitatively to lactic acid, and heterofermentative, which ferment glucose to lactic acid, ethanol or acetic acid and CO_2 (Sharpe, 1979). In practice, a test for gas production from glucose will distinguish between the groups (Sharpe, 1979). Leuconostoc and a subgroup of lactobacilli are heterofermentative; all other LAB are homofermentative.

Growth at certain temperatures is mainly used to distinguish between some of cocci. *Enterococci* grow at both 10°C and 45°C, *Lactococci* and *Vagococci* at 10°C but not at 45°C. *Streptococci* do not grow at 10°C, while growth at 45°C in dependent on the species. Salt tolerance (6.5% NaCl) may also be used to distinguish among *Enterococci*, *Lactococci*, *Vagococci* and *Streptococci* although variable reactions can be found among *Streptococci* (Mundt, 1986). Extreme salt tolerance (18% NaCl) is confined to the genus *Tetragenococcus*. Tolerance to acid and/or alkaline conditions are also useful characteristics. Enterococci are characterized by growth at both high and low pH. The formation of the different isomeric forms of lactic acid during fermentation of glucose can be used to distinguish between Leuconostocs and most heterofermentative Lactobacilli, as the former produce only D-lactic and latter a racemate (DL-lactic acid).

A summary of the identification of the LAB genera with classical phenotypic tests is shown in Table 2.1. The newly described genus *Carnobacterium* is distinguishable from *Lactobacillus* with this tests, as is *Vagococcus* from *Lactococcus*. *Vagococcus* and *Carnobacterium* have a unique fatty acid composition, which clearly separates these genera from other LAB. Furthermore, species of *Vagococcus* are generally motile (Collins *et al*., 1987, 1989), which is an unusual property among LAB. A typical character of carnobacteria is the failure to grow on acetate media, commonly used as selective media for lactobacilli (Collins *et al*., 1987), but this property is actually shared with some species and strains of lactobacilli. Pediococci can be confused with aerococci, since the morphology is similar. However, most pediococci are more acid-tolerant than aerococci and grow well anaerobically, contrary to the microaerophilic nature of aerococci. A former species of *Pediococcus*, *P. urinaeequi*, earlier the most likely to be confused with aerococci, is now considered to be *A. Viridans*, a suggestion mainly based on rRNA sequence (Collins *et al*., 1990). It should be noted that there are phenotypic overlaps between genera and exceptions to the general rules in Table 2.1 can be found. Classification of LAB is becoming dependent on more sophisticated methods, of which rRNA sequencing probably is the most accurate at the genus level.

2.1.3 Classification at Species Level

Classification of LAB is beginning to rely on molecular biology methods. This is perhaps truer regarding classification at species level at genus level. In some cases, only DNA-DNA homology studies can resolve problems in classification (Kandler, 1984). Still, the classical phenotypic or biochemical characterization is important for a preliminary classification as well as learning about the properties of the strains. Some of the characters listed in Table 2.1 are useful also in the classification at species level, e.g., salt and pH tolerance, growth at certain temperature and configuration of the lactic acid produced. Other characters used in the phenotypic or biochemical characterization of strains are range of carbohydrates fermented, arginine hydrolysis, acetoin formation (Vogues-Proskauer test), bile tolerance, type of hemolysis, production of extra-cellular polysaccharides, growth factor requirements, presence of certain enzymes (e.g., β-galactosidase and β-glucoronidase), growth characteristics in milk and serological typing. Further characterization includes more molecular or chemotaxonomic approaches, including type of diamino acid in the peptidoglycan, presence and type of teichoic acid, presence and type of menaquinones, guanine+cytosine (G+C) ratio of the DNA, fatty acid composition and electrophoretic mobility of the lactate dehydrogenase (LDH).

2.1.3.1 *Enterococcus, Lactococcus, Streptococcus* and *Vagococcus*

For details regarding the major taxonomic revision of the “streptococci” and the current identification of enterococci, lactobacilli and streptococci which summarized the phenotypical, biochemical and molecular characteristics of species included in the genera. The summary presented here is based on that review. Historically, serological typing with the Lancefield grouping (Lancefield, 1993) has been very important in the classification of streptococci. The method is now considered to be less important in classification, but still very useful in the rapid identification of major pathogens (Sharpe, 1979; Hardie, 1986b; Schleifer and Klipper-Balz, 1987). However, there is undoubtedly a strong (but not 100%) correlation between presence of the group D antigen and enterococci (previously designated “group D streptococci” or “fecal streptococci”). Similarly, the group N antigen is correlated with lactococci (previously “group N streptococci” or “lactic streptococci”), but note that the vagococci also possess the group N antigen (Collins *et al.*, 1989). sources. However, as tried to emphasize, they have developed systems that will allow them to derive more energy from a rich medium than just a carbohydrate. One of these systems are the co-fermentations that have been mentioned several times. By using a substrate, otherwise, as an electron acceptor during carbohydrate fermentation, they indirectly derive some energy from that substrate that otherwise would be lost. The malo-lactic fermentation serves the same purpose, whatever the actual mechanism for energy transduction may be. The use of co-fermentations may also enable LAB to extend the range of fermentable substrates. For example, *Lb. plantarum* is unable to ferment either citrate or mannitol anaerobically. However, added together, both substrates are used, mannitol as the main energy sources and citrate as electron acceptor.

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Table 2.1 Differential Characteristics of Lactic Acid Bacteria[a]

Characteristics	**Rods**		**Cocci**						
	Carnob.	***Lactob.***	***Aeroc.***	***Enteroc***	***Lactoc.*** ***Vagoc.***	***Leucon.***	***Pedioc.***	***Strepto.***	***Tetragenoc.***
Tetrad form	-	-	+	-	-	-	+	-	+
CO_2 from glucose	-	±	-	-	-	+	-	-	-
Growth at 10°C	+	±	+	+	+	+	±	-	+
Growth at 45°C	-	±	-	+	-	-	±	±	-
Growth in 6.5% NaCl	ND[d]	±	+	+	-	±	±	-	+
Growth at 18% NaCl	-	-	-	-	-	-	-	-	+
Growth at pH 4.4	ND	±	-	+	±	±	+	-	-
Growth at pH 9.6	-	-	+	+	-	-	-	-	+
Lactic acid[c]	L	D,L,DL[e]	L	L	L	D	L,DL[e]	L	L

Source: Axelsson (1993)

[a] +, positive; -, negative; ±, response veries between species; ND, not determined.

[b] Test for homo- or heterofermentation of glucose; negative and positive denotes homofermentative and heterofermentative, respectively.

[c] Configuration of lactic acid produced from glucose.

[d] No growth in 8% NaCl has been reported.

[e] Production of D-,L- or DL-lactic acid varies between specie

Despite the formation of new genera. The genus *Streptococcus sensu stricto* is still very large and the classification is difficult. The genus is broadly divided into three groups: pyogenic, oral and "other" streptococci. Some anaerobic cocci, previously included in the genus as the group "anaerobic streptococci" (Hardie, 1986b), were shown to be unrelated to all other streptococci and have been excluded (Schleifer and Klipper-Balz, 1987). The pyogenic group contains some famous pathogen, e.g., *S. pyogenes*. Another pathogens, *S. pneumoniae*, was earlier also included in this group, but has been transferred to the oral group, which otherwise contains species mostly associated with the oral cavity of man and animals. Some of the oral streptococci, e.g., *S. mutans*, can be cuastaive agents of dental caries (Hardie, 1986c). As a general rule, the pyogenic streptococci are β-hemolytic, while the oral streptococci are α- or nonhemolytic. The only streptococcal species that is associated with food technology is S. thermophilus, which is used in the manufacture of yogurt (in coculture with *Lb. delbruckii* subsp. *bulgaricus*), *S. thermophilus* was included in the group other streptococci and Hardie (1986d), but could conceivably be include in the oral group, since a close relationship at the DNA level with *S. salivarius* has been established (Farrow and Collins, 1984; Hardie, 1980d). Farrow and Collins (1984) proposed that *S. thermophilus* should be considered a subspecies of *S. salivarius*, since DND-DNA homology values of greater than 70% were determined. Thus, the name *S. salivarius* subsp. *thermophilus* became valid and to knowledge still is. However, the suggestion was later rejected, since an investigation of a large number of strains revealed much lower DNA-DNA homology values for some strains. In addition, the large phenotypic differences would justify two separate species (Schleifer and Klipper-Balz, 1987).

Lactococci are intimately associated with dairy products, but out of the five species currently recognized (Williams *et al*., 1990) only one, *Lc. Lactis*, is actually used in dairy technology. Three subspecies can be distinguished: *Lc. Lactis* subsp. *lactis*, *Lc. Lactis* subsp. *cremoris* and *Lc. Lactis* subsp. *hordniae*. Only the first two are important in dairy manufacture. *Lc. Lactis* subsp. *lactis* includes species formerly designated *S. lactis* subsp. *lactis*, *S. lactis* subsp. *diacetylactis* and *Lactobacillus xylosus*. The latter illustrates the fact that morphology sometimes can be deceptive in classification of LAB; i.e., the distinction between cocci and rods is not always an easy task. *Lc. Lactis* subsp. *cremoris* includes species previously designated *S. cremoris* or *S. lactis* subsp. *cremoris*. *Lc. Lactis* subsp. *cremoris* is distinguished from *Lc. Lactis* subsp. *lactis* by the inability to (i) grow at 40°C, (ii) grow in 4% NaCl, (iii) hydrolyze arginine and (iv) ferment ribose (Schleifer *et al*., 1985). As noted, species of the newly described genus *Vagococcus* are easily confused with lactococci, differing mainly in fatty acid composition and motility (Collins *et al*., 1989).

Enterococci are not considered to be of particular importance in food technology. Some species, in particular *E. faecalis* (previously *S. faecalis*), can be opportunistic pathogens (Parker, 1987) and are, therefore, generally undersirable in food. However, preparations of *E. faecium* (previously *S. faecium*) and *E. faecalis* have been used as probiotics (Fuller, 1986; Tournut, 1989) and as silage inoculants (Seale, 1986). The probiotic approach is not farfetched, since the natural habitat of most enterococci is the intestine of man and animals (Mundt, 1986). The species of enterococci are differentiated mainly by carbohydrate fermentation patterns, arginine and hippurate hydrolysis and presence and/or type of menaquinones (Schleifer and Klipper-Balz, 1987). *E. faecium* has been of great value for general LAB research in being a model organism in physiological studies of , for instance, bioenergetics and membrane biology (Kashket, 1987; Maloney, 1990).

2.1.3.2 *Arecoccus*, *Pediococcus* and *Tetragenococcus*

Pediococci are important in food technology, in both a negative and positive sense. *P. damnosus* is a major spoilage organism in beer manufacture, since growth may lead to diacetyl or acetoin formation, resulting in a buttery taste (Garvie, 1986b). *P. acidilactici* and *P. pentosaceus* are used as starter cultures for sausage making and as silage inoculants (Seale, 1986; Hammes *et al*., 1990). Pediococci may also be constituents of the complex known as the nonstarter lactic acid bacteria (NSLAB), which is involved in the ripening of cheese (Fox *et al*., 1990; Olson, 1990). The main characters for distinguishing between the species are the range of sugars fermented, hydrolysis of arginine, growth at different pH levels (7.0 and 4.5) and the configuration of lactic acid produced. *P. pentosaceus* and *P. acidilactici* cannot be separated using these characteristics, but have been shown to distinct species with DNA-DNA homology studies. These species are also similar in that they may produce a non-heme pseudocatalase (Whittenbury, 1964; Garvie, 1986b). Pediococci may be confused with aerococci. The genus *Aerococcus* is monospecific (*A. vividans*) and will not be dealt with further.

As memtioned, the genus *Tetragenococcus* contains strains previously regarded as *P. halophilus*, is currently recognized (Collins *et al*., 1990). In addition to its extreme salt tolerance (>18%NaCl), which distinguish it from other LAB, *T. halophilus* has a salt requirement for growth, generally 5% NaCl (Garvie, 1986b). *T. halophilus* is an important species in lactic fermentations of foods containing high concentrations of salt, e.g., soy sauce (Abe and Uchida, 1989).

2.1.3.3 *Leuconostoc*

The leuconostocs are separated from other cocci of the LAB by their heterofermentative metabolism. They can easily be confused with “coccoid rods” of the heterofermentative lactobacilli. The configuration of the lactic acid formed separate leuconostocs from most heterofermentative lactobacilli, but some species of the latter may also produce D-lactic acid (Kandler and Weiss, 1986). *Leuconostoc* are also unable to hydrolyze arginine, contrary to most heterofermentative lactobacilli, but there are exceptions among the latter. A number of investigations have been made concerning the relationship between leuconostocs and the species of heterofermentative lactobacilli that resemble the leuconostocs phenotypically (Garvie, 1986a; Yang and Woese, 1989; Martinez-Murcia and Collins, 1990). The current view is that *Ln. paramesenteroides*, together with some heterofermentative lactobacilli, e.g., *Lb. confurus* and *Lb. viridescens*, may represent the nucleus of a new genus, separated from both other heterofermentative lactobacilli and other leucnostocs (Martinez-Muricia and Collins, 1990). However, it is present not possible to distinguish between the *Ln. paramesenteroides* group and the main group of leuconostocs (designated *Leuconostoc sensu stricto*) using physiological or chemotaxonomic characters.

Leuconostocs may form significant amounts of diacetyl from citrate in milk and some species, mainly *Ln. mesenteroides* subsp. *cremoris,* have been used in the dairy industry for this purpose. Leuconostocs are also important in spontaneous vegetable fermentations, e.g., sauerkraut, where they often initiate the lactic fermentation (Daeschel *et al*., 1987). Species and subspecies are distinguished by characteristics such as carbohydrate fermentation patterns, dextran formation (from sucrose), growth requirements, salt and pH tolerance and dissimilation of citrate and/or malate, but classification is difficult (Garvie, 1986a). The dissimilation of malate requires attention, as this metabolism results in L-malic acid (Radler, 1975). Hence, care must be taken that malate is not present in the growth medium when the configuration of the lactic acid (from glucose fermentation) is to be determined. The malate-dissimilating, ethanol and acid-tolerance species, *Ln. oenos,* characteristically found in wine, is phenotypically very difficult from other leuconostocs. Phylogenetic investigations indicate that *Ln. oenos* may warrant a separate genus.

2.1.3.4 *Lactobacillus and Carnobacterium*

The genus 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. The heterogeneity is reflected by the range of mol% G+C of the DNA of species included in the genus. This range is 32-53%, which is twice the span usually accepted for a single genus (Schleifer and Strackebrandt, 1983). The heterogeneity and the large number of species are due to the definition of the genus, which essentially is rod-shaped lactic acid bacteria. Such a definition is comparable to an arrangement where all the coccoid LAB were included in one genus. However, among the cocci, phenotypic traits were early recognized, which made differentiation into several genera possible. Even if the situation was more difficult for the rod-shaped LAB. Orla-Jenson (1919) essentially tried to divide this group in a similar way as with the cocci. Thus, the subgenera of *Lactobacillus* were created:

Thermobacterium, *Streptobacterium* and *Betabacterium*. Remarkably, this division is still valid to a considerable degree, although the designations have been dropped and some modifications of the definition of the subgroups have been made (Kandler, 1984; Kandler and Weiss, 1986).

The classical ways of distinguishing among species of lactobacilli have been carbohydrate fermentation patterns, configuration of lactic acid produced, hydrolysis of arginine, growth requirements and growth at certain temperatures (Sharpe, 1979, 1981). These characters are still useful but, proper classification may also require analysis of the peptidoglycan, electrophoretic mobility of the LDH, mol% G+C of the DNA and even DNA-DNA homology studies. Lactobacilli are widespread in nature and many species have found application in the food industry. They are generally the most acid-tolerant of the LAB (Kashket, 1987) and will terminate many spontaneous lactic fermentations such as silage and vegetable fermentations (Daeschel *et al*., 1987). Lactobacilli are also associated with the oral cavity, gastrointestinal tract and vagina of human and animals. Some species, e.g., *Lb. brevis* and *Lb. plantarum*, can be found in many habitats. Others are more specialized and are found only in certain niches, e.g., the sourdough organism *Lb. sanfrancisco* and the yogurt-associated *Lb. delbrukii* subsp. *bulgaricus* (previously *Lb. bulgaricus*).

Species of the genus Carnobacterium were originally classified as group III lactobacilli under the designations *Lb. divergens*, *Lb. carnis* and *Lb. piscicola*. Later studies showed that these bacteria were separate from lactobacilli and warranted a separate genus (Collins *et al*., 1987) and that the metabolism of glucose was predominantly homofermentative (De Bruyn *et al*., 1988). The fatty acid composition of carnobacteria differ from lactobacilli. In addition, the combination of meso-diaminopimelic acid-direct-type peptidoglycan and produvtion of predominantly L-lactic acid, is shared by only a few species of lactobacilli (Kandler and Weiss, 1986). *Carnobacteria* are characteristically found in meat and meat products, where they are able to proliferate even at low temperatures. A simple identification key, confirmed by DNA-DNA hybridization, for distinguishing among *C. divergens*, *C. piscicola* and typical meat-associated lactobacilli (e.g., *Lb. curvatus*, *Lb. sake* and *Lb. viridescens*) has been published (Montel *et al*., 1991).

2.2 The benefits of Lactic Acid Bacteria

Lactic Acid Bacteria have a number of well-established benefits. They can improved lactose digestion, play a role in preventing and treating diarrhea and act on the immune systems, helping the body to resist and fight infection. More work needs to be done to confirm the role lactic acid bacteria might play in preventing or slowing the growth of colon cancer, lowering cholesterol levels, preventing urogenital infections, alleviating constipation and treating food allergy.

2.2.1 Lactose digestion

It is well-known that the presence of lactic acid bacteria, specifically *L. bulgaricus* and *S. thermophilus* in yoghurt, improves lactose digestion (Martini *et al*., 1991). It appears that the cell walls of the bacteria have to be intact (as is the case when the bacteria are alive) for the effect to occur (Kuhn *et al*., 1996). Some possible mechanisms for the improved lactose digestion include: The lactase activity of the bacteria actually does the work of digesting lactose in the product once it reaches the intestine (Martini *et al*., 1987). The slower transit time of yoghurt may permit more time for the residual intestinal lactase and the yoghurt bacteria to digest the lactose (Schaafsma, 1993). Something in the yoghurt may inhibit fermentation of lactose and thus reduce symptoms (Schaafsma, 1993). Sweet acidophilis milk (milk *L. acidophilus* which gas not been allowed to ferment) does not seem to alleviate the symptoms of lactose maldigestion. Although some work shows a small effect, most work shows no effect (Onwulata *et al*., 1989).

2.2.2 Diarrhea

Lactic acid bacteria may be useful in preventing and shortening the duration of several types of diarrhea (Sanders, 1994). A number of well-designed studies have noted that fermented milk products effectively prevent or treat infantile diarrhea (Saloff-Coste, 1995). Effects have been noted with *L. casei* and *B .bifidum*. A few small studies show that lactic acid bacteria can reduce the incidence of antibiotic-related diarrhea (Salminen and Deighton, 1992). This suggests a role for lactic acid bacteria in immuno-suppressed patients who routinely use antibiotics (Aronsson *et al.*, 1987). A few studies of traveler's diarrhea have demonstrated the effectiveness of lactic acid bacteria in decreasing the incidence of diarrhea (Black *et al*., 1989 and Oksanen *et al*., 1990).

Lactic acid bacteria can probably reduce diarrhea in several ways; Lactic acid bacteria compete with pathogens for nutrients and space in the intestines (Salminen and Deighton, 1992). By-products of metabolism may have a direct effect against the pathogens. For example, in vitro work shows that *L. casei*, *L. acidophilus* and *L. bulgaricus* can all produce antimicrobial agents such as acidophilin and bulgarican that can inhibit growth of pathogens (Salminen and Deighton, 1992). Lactic acid bacteria may be effective against diarrhea due to effects on the immune system.

2.2.3 Effects on the immune system

Lactic acid bacteria enhance immune system function at the intestinal and systemic levels. In humans, lactic acid bacteria have been shown to increase: B-lymphocytes or B cells, which recognize foreign matter (De Simone *et al*., 1993). Phagocytic activity, helping to destroy foreign matter (Schiffrin *et al*., 1995). IgA-, IgG- and IgM-secreting cells and serum IgA levels, which would increase antibody activity (Kaila *et al*., 1992). γ-interferon levels, which help white blood cells fight disease (Halpern *et al*., 1991).

Another way the body’s defenses work is by the barrier provided by the mucus layers of the intestine. The mucosa provide a physical barrier, usually preventing foreign substances from passing through the gut. As well, a large variety of immune cells are found in the gut mucosa. This allows the gut to interact with the immune system. Lactic acid bacteria can stimulate immune activity in the intestinal mucosa (Perdigon *et al.*, 1993). In conditions such as allergy or auto-brewery syndrome (abnormal gut fermentation resulting in increased levels of blood ethanol), the permeability of the small intestine can increase, allowing undigested protein molecules to pass through (Joneja *et al.*, 1997). Lactobacillus GG has been shown to reverse gut permeability (Isolauri *et al.*, 1993). Probiotic bacteria may be able to play a role in treating food allergy. This was demonstrated in a recent experimental with infants known to have excema due to a cow milk allergy (Majamaa and Isolauri, 1997). Infants in the experimental group got hydrolyzed whey formula fortified with LGG, while those in the control group just got whey formula. The skin condition of the infants getting the LGG improved significantly compared to the control group. In addition, the experimental group had improved levels of factors associated with inflammation of the intestine.

2.2.4 Potential benefits Hypo-cholesterol effects

Some strains of *L. acidiphilus* can take up cholesterol in the presence of bile (Buck and Gilliland, 1994). Other in vitro research showes that cholesterol can precipitate with free bile salts in the presence of *L. acidophilus*, especially in an acid environment (Klaver and van der Meer, 1993). Thus, it has been hypothesized that one or both of these actions would take place in vivo and help lower serum cholesterol in humans. Various studies with fermented milk products have shown either no effect or a reduction in cholesterol levels. In conclusion, there is not yet good evidence to confirm a cholesterol-lowing effect of fermented milk products.

2.2.5 Constipation

Milk products fermented with some strains of *L .acidophilus* and *bifidobacterium* shorten intestinal transit time. This effect may be useful for those with constipation, such as the elderly (Hitchins and McDonough, 1989). A well-controlled human study is need to confirm this.

2.2.6 Colon cancer

Several lactic acid bacteria may help prevent initital of colon cancer. It has also been demonstrated that lactic acid bacteria sloe the growth of experimental cancers, although the results are not long-term. It appears that lactic acid bacteria can reduce the levels of colon enzymes that convert procarcinogens to carcinogens. Specifically, lactic acid bacteria can reduce levels of the enzyme β-glucuronidase, nitroreductase and azoreductase. Lactic acid bacteria may also be involved in the direct reduction of procarcinigens for example, by taking up nitrites and by reducing the levels of secondary bile salts (Fernandes and Shahani, 1990). In most reports, these effects only occur during the period of time that the bacteria are consumed (Marteau *et al*., 1990). Changes in enzyme activity in humans have been observed with *L. acidophilus* and *B. bifidum* and LGG (Goldin and Gorbach, 1984). Animal studies show fewer tumors in those exposed to a carcinogen, in the presence of LGG, compared to the animals exposed to carcinogen without the benefit of LGG (Goldin, 1996). In humans, epidemiological reports show that populations eating fermented dairy products have a decreased risk of colon cancer (Kampman *et al*., 1994). However, there is not yet a clear relationship between lactic acid bacteria intake and cancer prevention.

2.2.7 Urogenital infections

Lactic acid bacteria may reduce candidal vaginal infections. This is still speculative, however it would be research worth pursuring. One small study showed that women with recurrent vaginal candidiasis who ate 9 oz. Daily of a yoghurt containing *L. acidiphilus* had fewer occurrences of vaginal candidiasis than during the control period in which they ate no yoghurt (Hilton *et al*., 1992). This was a cross-over study which started with 21 women. Eight of those who started in the treatment group refused to cross over to the control phase since they experienced so many fewer infections. Thus only 13 women completed the study.

2.2.8 Ulcers

Lactic acid bacteria show some promise against stomach ulcers. Work with a specific strain of *L. acidophilus* demonstrated that *L. acidophilus* competes effectively (in vitro) against *Heliobacter pylori* for attachment sites, limiting the number of *H. pylori* that can attach to the cell wall (Brassart *et al*., 1995). Infection with *H. pylori* is a risk factor for stomach ulcers. A small study of patients with ulcers showed that *Bifidobacteria bifidum* promoted healing of gastric ulcers in 50% of the patients and eradication of H. pylori from the mucous membranes in 30% of the patients (Saminen and Tanaka, 1995).

2.3 Lactic Acid Bacteria in Industry

Since the early 1900s there has been a marked worldwide increase in the industrial production of cheeses and fermented milks. Process technology has progressed toward increasing mechanization, increase in factory sizes, shortening processing times and larger quantities of milk processed daily in the factory. Milk can be fermented to more than 1,000 products with demands of their own special flavor, texture and final product quality. All this is reflected in enormous demands at the starter cultures, their activity, stable quality and bacteriophage resistance. The art of making cultured food products by using the former days whey or ferment products for today’s process has been changes to a science with exact knowledge of the factors influencing the specific starter species and strains.

During the 1980s many outstanding reviews were published about the metabolism, physiology, genetics, production and use of starter cultures. Thus, in this chapter the general view of the most important factors concerning starter activity, its effect on the final product and industrial starter production are discussed. The discussion of industrial production of starters is mostly based on the author's experience.

2.3.1 Use of starters in the dairy process

There has been considerable changes in the cultivation of starters during the past 20 years at the dairy level. The starter available for the dairy processes are solid in different forms by several starter producers. Starters are the most important factor determining the final quality and properties of the product. Therefore, the selection of starter type and form for use is very important for the individual dairy plant.

2.3.2 Starter cultures for fermented meat vegetable products

The growth of lactic acid bacteria in milk to produce fermented dairy products is based on a few simple principle: lactic acid bacteria are present, among other bacteria, in several nitches. When the physical conditions (such as temperature, AW, pH) allow growth, there is a competition between the various species. The faster development of lactic acid bacteria and the pH decrease by acid production lead to microbiologically stable fermented products. These basic phenomenon of bacterial ecology have also been used to produce meat products such as sausages and fermented vegetables.

Table 2.2 Some of the major lactic acid bacteria used in fermented milk products

Bacteria	**Products**
Thermophillic, homofermentative *Lactobacillus* *Lb. delbrueckii* ssp. *bulgaricus* *Lb. delbrueckii* ssp. *lacits* *Lb. acidophilus* *Lb. fermentum*	Yogurt, Swiss and Italina cheeses, Bulgarian buttermilk, yogurt drink, koumiss , acidophilus milk, miru-miru, kefir
Mesophilic, heterofermentative *Lactobacillus* *Lb. casei* ssp. *casei* *Lb. plantarum* *Lb. brevis* *Lb. kefir*	Yakult, yogurt drink, miru-miru, kefir
Mesophillic *Streptococcus* (*Lactococcus*) *Str. lactis* ssp. *lactis*	Scandinavian fermented milk, cultured butter milk, cultured cream, kefir
Thermophilic *Streptococcus* (*Lactococcus*) *Str. Thermophilus*	Yogurt
Leuconostoc	
Leu. mesenteroides ssp. *mesenteroids*	Kefir
Bifidobacterium	
Bif. bifidum	Fermented milk
Bif. logum	Fermented milk
Bif. breve	Fermented milk, miru-miru
Bif. infantis	Fermented milk

Source: Mitsuoka, 1990

For sausage preparation, beside the meat slurry, fermentable sugars, salt and species, pediococci, such as *P. acidolactici*, *Lb. plantarum* and/or *Staphylococcus carnosus* are generally inoculated. The first incubation period (time and temperature are adapted to the various technologies) allows the bacteria to grow and a pH decrease is measured. After that sausages are cooled and ripened to obtain the final product. The widely available fermented vegetables are sauerkraut, pickled cucumbers and green olives. Most of the time, cabbage, cucumbers or olives are fermented with *Lb. plantarum*. Also *P. cerevisiol*, *P. pentosaceus*, *Leuconostoc mesenteroids* or *Lb. brevis* can be used (Boisen, 1981). During the preparation of these products, NaCl brine is added, the combination of NaCl and lactic acid makes the fermented vegetables stable for a long period of time, at room temperature. The salinity also controls the growth of lactic acid bacteria, thereby influencing the quality of the end product.

2.4 Human Gastrointestinal tract.

The human digestive system is a complex series of organs and glands that processes food. In order to use the food we eat, our body has to break the food down into smaller molecules that it can process; it also has to excrete waste. Most of the digestive organs (like the stomach and intestines) are tube-like and contain the food as it makes its way through the body. The digestive system is essentially a long, twisting tube that runs from the mouth to the anus, plus a few other organs (like the liver and pancreas) that produce or store digestive chemicals.

The digestive process begins in the mouth. Food is partly broken down by the process of chewing and by the chemical action of salivary enzymes (these enzymes are produced by the salivary glands and break down starches into smaller molecules). After being chewed and swallowed, the food enters the esophagus. The esophagus is a long tube that runs from the mouth to the stomach. It uses rhythmic, wave-like muscle movements (called peristalsis) to force food from the throat into the stomach. This muscle movement gives us the ability to eat or drink even when we're upside-down.

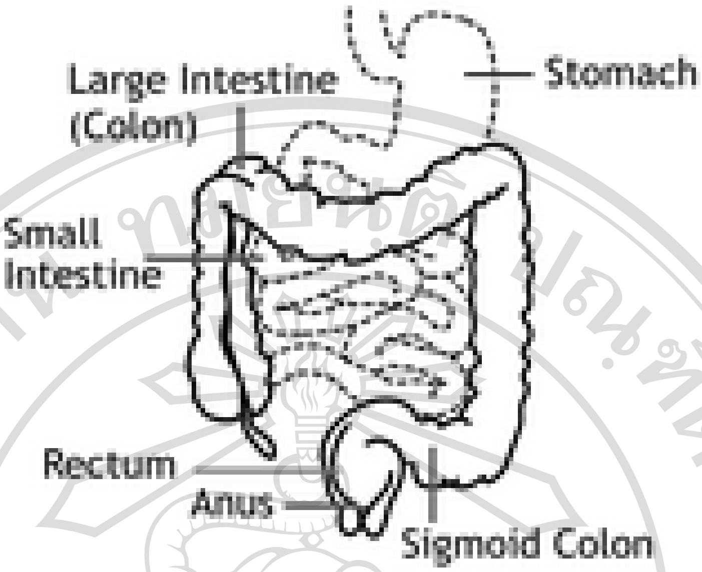

Figure 2.1 Human intestinal tract.

In the stomach; the stomach is a large, sack-like organ that churns the food and bathes it in a very strong acid (gastric acid). Food in the stomach that is partly digested and mixed with stomach acids is called chyme. The pH of the stomach in a normal, healthy human is in the 1-3 range. There are many purposes for the high acidity found in the stomach. One of the main functions of the high acid content is to destroy bacteria that are ingested. Few bacteria can survive in an environment with a pH of 1 to 3, because of an impenetrable outer coat that can resist acid breakdown. Another purpose for such a low pH is that high acidity is required to activate pepsinogen. Pepsinogen is the enzyme that initiates the digestion and breakdown of proteins that are ingested. The other major component of gastric fluid is mucus. Mucus provides protection to the stomach lining from the high acid content. Gastric pH varies from time to time. Gastric acid is secreted in anticipation of a meal, to prepare for digestion. Gastric pH decreases as a result of acid secretion, and, after a heavy meal, blood pH correspondingly increases, particularly in those segments of the circulatory system associated with supplying the gastrointestinal tract. This increase in blood pH is known as the "alkaline tide", and is caused by bicarbonate ions that are secreted into extracellular fluid of the stomach, then into venous blood.

In the small intestine; bile (produced in the liver and stored in the gall bladder), pancreatic enzymes, and other digestive enzymes produced by the inner wall of the small intestine help in the breakdown of food. After being in the stomach, food enters the duodenum, the first part of the small intestine. The pH of the duodenum is 6 to 6.5. The majority of nutrients, vitamins, and drugs are absorbed in this 6 inch area of the gastrointestinal tract. In addition to water, mucus, and electrolytes, secretions from the liver and pancreas join secretions from the intestinal mucosa to facilitate digestion and absorption. The anatomy of the small intestines is such that a very large surface area that provides better absorption is available. The lining of the small intestines is composed of many villi, or finger like projections, which extend even more as projections called the brush border. The area is highly perfused with blood. These factors contribute to a very high surface area, increasing the likelihood of drug absorption taking place, if the ionization criterion is met. The pH can reach 7 to 8 in this area. Further along the small intestine, beyond the duodenum, lies the jejunum and ileum. These sections of the small intestine lack the high surface area of the duodenum and only small amounts of absorption across the lipid membranes occurs in this section of the small intestine. As we get further away from the stomach, the pH rises to about 7.5 in this region.

In the large intestine; after passing through the small intestine, food passes into the large intestine. Some of the water and electrolytes (chemicals like sodium) are removed from the food. Many microbes (bacteria like *Bacteroides*, *Lactobacillus acidophilus*, *Escherichia coli*, and *Klebsiella*) in the large intestine help in the digestion process. The first part of the large intestine is called the cecum (the appendix is connected to the cecum). Food then travels upward in the ascending colon. The food travels across the abdomen in the transverse colon, goes back down the other side of the body in the descending colon, and then through the sigmoid colon. The end of the process; solid waste is then stored in the rectum until it is excreted via the anus. The pH of the large intestine is 5.5-7.

2.5 Lactic Acid Bacteria in human intestine.

The human intestinal tract is inhabited by a diverse bacterial population that constitute a complex ecosystem. More than 400 different bacterial species have been isolated and identified. Strictly anaerobic bacteria are the most common organism in the intestinal tract more over facultative bacteria.

In table 2.3 show the most prevalent microorganism found of various locations in the human gastrointestinal tract. The stomach and upper small intestine have relatively low numbers of microorganisms. In the lower ileum, the number of bacteria increase to between 10^6-10^8. In the colon, the bacterial concentration increase to between 10^{11}-10^{12} organism per milliliters of fecal material and some of them were LAB, example *Streptococcus, Lactobacillus and Bifidobacterium.*

The intestinal microflora of healthy humans is confined to the distal ileum and the colon. It is comprised of numerous species of bacteria, most of which are obligately anaerobic and some of which attain high population levels. According to the results of cultures studies, thirty to forty bacterial species constitute 99% of the cultivable fecal microflora of a healthy human. Some of these species are present at populations of at least 10^{10} per gram of feces (wet weight). Even with optimal anaerobic conditions of sample processing and culture, however, the total colony forming units (CFU) count represents 88% of the total bacterial cell count obtained by microscopy.

This means that many of bacterial cells seen with the microscope are dead, or that many bacterial cells are alive but in physiological state that renders them non-culturable, or that many bacterial types cannot be cultured by conventional techniques. A similar situation has been encountered in microbiological studies of environmental samples where a relatively small proportion of the microflora is in a culturable state. Research of the intestinal microflora have been aware of this situation for some time, but it is only recently that technological advances have permitted the use of alternative methods for the analysis of microbial communities. The results of nucleic acid–based techniques now allow a more complete appraisal of the intestinal microflora to be made and the use of this technology will result in a renaissance of interest in the intestinal microflora.

Some of lactic acid bacteria are believed to function as probiotis. A probiotic is a culture of living microorganisms, mainly lactic acid bacteria or bifidobacterium, which beneficially affects the health of the host when ingested in sufficient quantities. The colonization of the gut by probiotic bacteria prevents the growth of harmful bacteria by competitive exclusion and by the production of organic acids and antimicrobial cmpounds (Fuller, 1989; Mogenson, 1995). Salminen, Deighton, Benno and Gorbach (1998) have recently reviewed the important properties and selection criteria for probiotica as well as providing examples of the practical effects of probiotics on intestinal infections, the maintenance of the indigenous microflora and even the alleviation of lactose intolerance. In order to act as a probiotic in the gastrointestinal tract the bacteria must be able to survive the acidic conditions in the stomach and resist the bile acids at the beginning of small intestine (Holzapfel *et al*.,1998; Molly *et al*., 1996 and Salminen *et al*., 1996). It is said that approximately 1% of the organisms in a fermented product survive through the gastrointestinal tract. However, this varies a lot depending on the strain used and the conditions of administration (Mogenson, 1995). The survival of bacteria in gastric juice depends on their ability to tolerate low pH. The pH of excreted HCl in stomach is 0.9. Whatever, the presence of food raises the pH value to the level of pH 3. After the ingestion of food it takes 2-4 h for the stomach to empty. Bile salts are systhesized from cholesterol in the liver, stored in the gall bladder, and released into the small intestine after ingestion of a fatty meal. This detergent is critical to microorganisms since their cell membranes are composed of lipids and fatty acids. However, some microorganisms are able to hydrolyze bile salts with bile salt hydrolase enzyme (BSH) decreasing their solubility, and thus weakening their detergent effect (Hofmann and Mysels, 1992). BSH activity has been found in many species including *Lactobacillus* (Gilliland and Speck, 1997).

Table 2.3 Distribution and composition of the intestinal flora.

Site	Composition	Total number of organisms per ml. contents
Stomach	*Streptococcus* *Lactobacillus*	10^1-10^2
Duodenum and jejunum	Similar to stomach	10^2-10^4
Ileal and cecal	*Bacteroides* *Clostridium* Streptococci Lactobacilli	10^6-10^8
Colon	*Bacteroides* (10^{10}-10^{11}) *Clostridium* (10^{10}) *Eubacterium* (10^{10}) *Peptococcus* (10^{10}) *Bifidobacterium* (10^9-10^{10}) *Streptococcus* (10^{10}) *Fusobacterium* (10^9-10^{10})	10^{11}-10^{12}

Source: Moore and Holderman (1974)

2.6 Probiotic Lactic Acid Bacteria.

2.6.1 Probiotic concept

The term “probiotic” was introduced by Lilly and Stillwell in 1965 for growth promoting factors produced by microorganisms. The word “probiotic” is derived from Greek and means “pro life”. In 1974, Parker used the term for organisms and substances is imprecise and would include even antibiotics. Therefore, in 1989 Fuller defined “probiotic as a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance”. However, according to this definition probiotics were restricted to feed supplements, animals and the intestinal tract, and the term probiotic thus could not be used for living microorganisms administered in any other way than in food or feed, or for locations other than the gastrointestinal tract. Consequently, in 1992 Havenaar and Huis in’t Veld proposed to broaden Fuller’s definition into a probiotic is a mono or mixed culture of live microorganisms which, applied to animal or man, affect beneficially the host by improving the properties of the indigenous microflora.

So, probiotic by the general accepted definition, is a “live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance”. Although referring to the supplementation of animal feeds for farm animals, the definition is easily applied to human situation. The major consumption of probiotics by humans is in the form of dairy-based foods containing intestinal species of lactobacilli and bifidobacteria. It is implicit in the definition that consumption of the probiotic affects the composition of the intestinal microflora. This effect of the probiotic on the intestinal ecosystem, it is proposed, impacts in some beneficial way on the consumer. Most probiotics contain Lactic Acid Bacteria because of they are components of natural microflora of almost all organisms. They rarely pathogenic and present against pathogenic microorganisms.

2.6.2 The properties of Probiotic Lactic Acid Bacteria.

Probiotics strains can be used only, and are active only, on or in the body of the host if they fulfill a large number of criteria. The criteria for the selection and assessment of probiotic microorganisms were the result of the collaboration of research institutions and universities with food industries. The list of properties expected from potential probiotic strains of lactic acid bacteria have been proposed including:

- increasing resistance to infectious diseases, particularly of the intestine
- decreased duration of diarrhea
- reduction in blood pressure
- reduction in serum cholesterol concentration
- reduction in allergy
- stimulation of phagocytosis by peripheral blood leucocytes
- modulation of cytokine gene expression
- adjavant effect
- regression of tumours
- reduction in carcinogen or co-carcinogen production

Several basic properties are required for an effective probiotic strain of lactic acid bacteria. Among the most important properties is the ability to survive passage through the mouth, stomach, small intestine and large intestine. Thus, the strains must be stable in gastric conditions and resistant to acid. In addition, stability to bile acid is required. In order to be able to influence functions of the human intestinal tract, probiotic strains must have capacity to adhere to intestinal mucosal cells and to grow in intestinal conditions (Table 2.4).

Table 2.4. Characteristics expected of potential probiotic strains

Number	Characteristics
1	Accurate taxonomic identification
2	Normal inhibitant of the species targeted: human origin for human probiotics
3	Nontoxic and nonpathogenic
4	Genetically stable
5	Capable of survival, proliferation, and metabolic activity at the target site
6	Adherence and colonization potential preferred
7	Stability of desired characteristics during culture preparation, storage, and delivery
8	Viability at high populations preferred at 10^6-10^8
9	Production of antimicrobial substances including bacteriocins, hydrogen peroxide and organic acids
10	Antagonistic toward pathogenic/carioganic bacteria
11	Able to complete with the normal microflora including the same or closely related species; potentially resistant to bacteriocins, acids, and other antimicrobials produced by residing microflora
12	Resistant to bile
13	Resistant to acid
14	Immunostimulatory
15	Able to exert one or more clinically documented health benefits
16	Amenable to production processing: adequate growth, recovery, concentration, freezing, dehydration, storage, and distribution
17	Provision of desirable organoleptic qualities (or no undesirable qualities) when included in fermentation processes

Source: Crowell (1998)

2.6.3 Lactic Acid Bacteria as Probiotics

Most probiotic microorganisms belong to Lactic Acid Bacteria (LAB), such as *Lactobacillus* sp, *Bifidobacterium* sp and *Enterococcus* sp (Klein *et al*., 1998). The yeast Saccharomyces boulardii has been studied extensively (Elmer *et al*., 1999) and also other bacterial species, like *Bacillus* sp (Senesi *et al*., 2001) and *Clostridium butyricum*. In some countries the use of *Enterococcus* sp as a proboitic has been questioned because of safety aspects with regard to transfer of genus conferring antibiotic resistance (Lund and Edlund, 2001). Most scientist agree that probiotic strains shall be able to survive transit through the gastric acid environment as well as exposure to bile and pancreatic juice in the upper small intestine to be able to exert beneficial effects in the lower small intestine and the colon, although there are convincing data on beneficial immunological effects also from dead cells (Mottet and Michetti, 2005). Best effect is achieved if the microorganisms colonize the intestinal surface mucus layer since they then can affect the intestinal immune system, displace enteric pathogens, provide antioxidants and antimutagens, and possibly other effects by cell signaling. That intake of LAB influences multiple systems was elegantly shown for Lactobacillus GG using microarray analysis (Di Caro *et al*., 2005). One month treatment resulted in up-regulation of 334 genes and down-regulation of 92 genes involved in inflammation, apoptosis, cell-cell signaling, cell adhesion and differentiation and signal transcription and transduction.

In recent years, multiple reports have described beneficial effects from various aspects on important diseases, like intestinal infections, inflammatory bowel diseases (IBD), and allergy by addition of selected strains to food products, often together with fiber or a prebiotic substance. In many countries, there are now several probiotic products on the market but the documentation is often based upon case reports, animal studies or uncontrolled small clinical trials. Furthermore, there is no general acceptance on how to characterize probiotic microorganisms, and few products declare the actual content of live microorganisms.

2.6.4 Cell surface properties mediating adhesion

Mechanisms of adherence to an epithelial surface involve both receptor-specific binding and charge and hydrophobic interaction. LAB commonly express cell surface hydrophobicity (CSH) as measured by the Salt Aggregation Test (SAT), contact angle and adhesion to xylene (Wadstrom *et al*., 1987; Strus *et al*., 2001). Some LAB coaggregate with cells of the same strain or with cells from other species (Kmet *et al*., 1995; Roos *et al*., 1999; Kolenbrander, 2000). This mechanism may facilitate adhesion, e.g. to mucus. LAB can also express binding of extra-cellular matrix molecules (ECM), like collagens, fibronectin and vitronectin which may be shed from the epithelium to the mucus layer, and to mucus components (Aleljung *et al*., 1994; Sillanpaa *et al*., 1995; Howard *et al*., 2000; Lorca *et al*., 2002A). Strains of *L. acidophilus*, *L. gasseri*, *L. johnsonii*, *L. crispatus* and others form a Surface (S-) layer which covers the cell surface during growth and may contain substances which mediate adhesion to the intestinal surface (Sillanpaa *et al*., 1995; Smit *et al*., 2001; Ventura *et al*., 2002), or convey cell surface hydrophobicity (Vadillo-Rodriguez *et al*., 2005). S-layers show a high similarity of their amino acid composition but show small general sequence similarity (Avall-Jaaskelainen and Palva, 2005). It is also important to study adhesion to different parts of the gastrointestinal (GI) tract since adhesion is likely to differ between the different compartments (Ouwehand and Salminen, 2003). Interestingly, LAB strains showed no host specificity in adhesion to intestinal mucus from various hosts (Rinkinen *et al*., 2003).

2.6.5 Survival within the gastrointestinal tract

Lactobacillus sp and *Bifidobacterium* sp show a moderate tolerance to acid pH during 90 min incubation which is decreased after 2 h but individual strains vary considerably (Charteris *et al*., 1998). Acid tolerance can be mediated by membrane ATPases as described for *L. acidophilus* (Lorca and Font de Valdez, 2001), *B. lactis* and *B. animalis* (Matsumoto *et al*., 2004). In the presence of milk or other food products the resistance was significantly higher (Saxelin *et al*., 1999). Most LAB were susceptible to bovine and porcine bile *in vitro*. However, they were resistant to human GIT (Dunne *et al*., 2001). In *L. reuteri*, bile resistance appeared to be mediated by bile salt hydrolysis (De Boever *et al*., 2000). This also resulted in precipitation of cholesterol. Similar effects were seen after deconjugation of bile salts by *L. acidophilus* strains (Ahn *et al*., 2003; Asher and Prajapathi, 1998). These reactions could possibly be the mechanism behind a reported decrease of serum cholesterol in patients treated with probiotics (Agerbaek *et al*., 1995; Asher and Prajapathi, 2000) as well as an anticlerotic effect of *L. bulgaricus*. In *L. acidophilus* NCFM, two genes encoding bile salt hydrolysis were identified (McAuliffe *et al*., 2005). One of these, *bsh*A, showed significant similarity to bile salt hydrolases (BSH) of *L. johnsonii, B. longum* and *Listeria monocytogenes* (McAuliffe *et al*., 2005; Pridmore *et al*., 2004; Tanaka *et al*., 2000). However, the resistance to bile salts varies a lot between the Lactobacillus species and even between strains (Chateua *et al*., 1994; Gilliland *et al*., 1985 and Xanthopoulos *et al*., 1997) and the mechsnism is still unknown (Charteris *et al*., 1998). Gilliland, Staley and Bush (1984) considered 0.3% bile salts as a critical concentration to screen for resistant strains. Goldin and Gorbach (1992) stated that the same level should be critical when selecting human probiotics.

2.6.6 Influence of stress on LAB Probiotics

Cold shock-induced proteins in *Lactobacillus lactis* have been extensively studied but also to same extent in *L. acidophilus* and *L. plantarum* because of their use as dairy starter cultures (Lorca and Font de Valdez, 1999; Wouters *et al*., 2000; Derzelle *et al*., 2000). Several cold shock proteins belong to a family of RNA and DNA chaperones of about 7 kDa which stabilize single standard regions of RNA and DNA. However, in the GI tract bacteria are exposed to stress in the form of acid pH, bile and pancreatic juice. Exposure to pH 4.5 during 1 h induced de novo synthesis of 9 proteins, 4 of which cross-reacted immunologically with heat shock proteins (HSP) in *L. acidophilus, L. plantarum, L. paracasei, Leuconostoc mesenteroides* and *Pentococcus pentosaceus* (Lorca *et al*., 2002B; Kruszewska *et al*., 2002). At the gene level, 72 genes were induced during passage through the GI tract, 4 of which were involved in stress-related functions (Bron *et al*., 2004). In an interesting study on effects of acid, bile-salt and freezing stress, log-phase cultures of *Lc lactis spp lactis* adapted better to all three stress forms than *Lc lactis spp cremoris*. Stationary-phase cultures of both subspecies were quite resistant to all forms (Kim *et al*., 1999).

2.6.7 Production of antioxidants

Reactive oxygen species are produced during passage of nutrients through the GI tract. The natural production of host antioxidants decrease rostrally. It is well known that oxidative damage forms part in the pathogenesis of cancer, cirrhosis, atherosclerosis and other chronic diseases. *B. longum* ATCC 15708 and to a lesser extent *L. acidophilus* ATCC 4356 inhibited linoleic acid peroxidation and scavenged free radicals (Lin and Chang, 2000). In a recent study, oblingately homofermentative lactobacilli produced high antioxidant activity whereas this was highly strain dependent among facultatively and oblingately heterofermentative lactobacilli (Annuk *et al*., 2003).

2.6.8 Antimicrobial effects

LAB commonly produce bacteriocins which are peptides with bactericidal activity usually against strains of closely related species. Although bacteriocins may enhance survival of LAB in complex ecological systems interest has focused on prevention of growth of harmful bacteria in the fermentation and preservation of dairy products. It is therefore more interesting with respect to probiotics that individual strains may inhibit growth of or adhesion of pathogenic microorganisms by secreted products, and not merely an effect of acidic pH. The gastric pathogen *Helicobacter pylori* was inhibited by a protein secreted from *L. acidophikus*, and *Escherichia coli* 0157:H7 (EHEC) was eradicated in rumen fluid by feed supplement containing *S. cerevisiae* ssp *boulardii* (Lorca *et al*., 2001; Bach *et al*., 2003). Strains of *B. infantis* and *L. salivarius* isolated from healthy Korean infants exerted bactericidal activity against both Clostridium difficile and EHEC (Lee *et al*., 2003A). Out of 24 Bifidobacterium strains, six inhibited *H. pylori* through production of a heat-stable compound which was sensitive to protease treatment (Collado *et al*., 2005). *L. acidophilus* LB produced an extra-cellular substance which inhibited several enteropathogens *in vivo* and *in vitro*, including *H. pylori* and *Salmonella enterica* var. Typhimurium (Coconneir *et al*., 1998; Coconnier *et al*., 2000). Lactobacillus treatment also inhibited an adhesion-dependent IL-8 production by *S. enterica*. Three *Pediococcus* sp were shown to produce bacteriocins, sensitive to protease treatment but resistant to amylase and pepsin, with activity against several gram-negative and gram-positive bacteria, such as *Pseudomonas aeruginosa*, *Bac. Cereus* and *S. aureus* (Jamuna and Jeevaratnam, 2004). Feacal isolates from healthy Brazillian volunteer were screened for *in vitro* activity against *Vibrio cholerae* (Silva *et al*., 2001). One *Lactobacillus* sp and one *Peptostreptococcus* sp showed high in vitro activity, and eliminated vibrios from germ-free mice after bi-association. The broad spectrum antibacterial effect of *B. subtilis*, however, due to secretion of antibiotic substances (Pinchuk *et al*., 2001).

2.6.9 LAB Probiotics and the immune system

The mammalian intestine is inhabited by a dense and antigenically foreign microflora. Nonetheless, the intestinal immune system remains normally in a state of homeostasis, and in fact appears to ignore or otherwise tolerate most intestinal microbes. Tolerance may reflect advantages conferred by the indigenous microflora to the host. For example, intestinal bacteria provide the host with several nutrients, including short chain fatty acid, vitamin K, some B vitamins and amino acids. Intestinal bacteria also protect the host from pathogens, forming a front line of mucosal defense. Specifically, gut bacteria prevent intestinal colonization by pathogenic organisms directly by competing more successfully for essential nutrients or for epithelial attachment sites (Rolfe, 1996). Through the production of antimicrobial compounds, volatile fatty acid, chemically modified bile acids, gut bacteria also create a local environmental that is generally unfavorable for growth of many enteric pathogens. Finally, the indigenous microflora induces recruitment of lamina propria immune cells, which form a second tier of defense by activation of appropriate inflammatory or immune mechanisms during infection. In return for providing the host with nutritional benefits and enhanced defense against pathogens, the indigenous microflora gains access to a nutrient-dense, stable environment and thereby enters a mutualistic relationship with the host.

Because of the reported positive effects of intestinal bacteria on host health, including prevention of cancer, decreased blood cholesterol, prevention of diarrhea, enhanced immune function and improved animal growth, researchers and clinicians have attempted to enhanced these effects through the provision of beneficial, probiotic bacteria (Gaskins, 1996). However, in many cases claims have been made without rigorous scientific validation. The claims of probiotic-mediated immune enhancement based on present knowledge of interactions between the host immune system and the indigenous microflora. Specifically, the role of nonpathogenic bacteria in the development of the intestinal immune system and in protecting the host from pathogenic insults. The relative immunogenicity of various members of the indigenous microflora and consequences of “broken” immune tolerance to the indigenous bacteria are considered in a discussion of animal models, including GF animals with a defined microbiota and in rodent models of inflammatory disorders, including IBD. Finally, available data on interactions between the host and the intestinal microflora will be analyzed to consider the potential of probiotic therapy to module human or animal immune function.

2.6.10 LAB Probiotics for farm animals

The development of probiotics for farm animals is based on the knowledge that the gut microflora is involved in resistance to disease. The stressful conditions experienced by the young animal causes changes in the composition and/or activity of the gut microflora. Probiotic supplementation seeks to repair these deficiencies and provide the type of microflora which exists in feral animals uninfluenced by modern farm rearing methods. The products available are of varying composition and efficacy but the concept is scientifically-based and intellectually sound. Under the right conditions the claims made for probiotic preparations can be realized (Fuller, 1997). The belief that colonization and growth in the gut was a prerequisite of probiotic activity prompted the use of intestinal isolates like L. acidophilus by Rettger and his colleagues in the USA (Rettger et al., 1935). Subsequently, increased knowledge of the gut microflora showed that the lactic acid microflora was more complex than was formerly thought and led to the use of a wide variety of different genera such as Lactobacillus, Bifidobacterium, Streptococcus and Enterococcus. These group, together with Bacillus sp., yeasts and filamentous fungi are the main components of the probiotics commonly used for farm animals today (Table 2.5).

Table 2.5 Microorganisms used in Probiotics for Farm Animals

Microorganisms
Lactobacillus acidophilus
L. casei ss. Casei
L. rhamnosus
L. reuteri
L. plantarum
L. brevis
Streptococcus salivarius ss thermophilus
S. lactis
Enterococcus faecium
E. faecalis
Bifidobacterium bifidum
B. pseudolongum
B. brevis
B. thermophilus
Bacillus subtilis
B. cereus
Clostridium butyricum
Pediococcus pentosaceus
Saccharomyces cerevisiea
Aspergillus oryzae
Candida pintolopesii

Source: Fuller (1997)

Much of the information on the use of probiotics for farm animals is derived from experimental trails performed in-house or contracted out by commercial organizations producing or selling probitics. Few of these ever get published in peer-review journals and it is difficult to assess their importance without the detail that such publication would demand. However, the potential benefits that can arise from applications of the probiotic concept are shown in Table 2.6.

Table 2.6 Potential Beneficial Effects of Probiotics for Farm Animals

The benefits
Greater resistance to infectious diseases
Increased growth rate
Improved feed conversion
Improved digestion
Better absorption of nutrients
Provision of essential nutrients
Increased milk yield
Improved milk quality
Increased egg production
Improved egg quality
Improved carcass quality and less contamination

Source: Fuller (1997)

2.6.11 LAB Probiotica as live vaccines

Although LAB offer many potential advantages as live vaccine vehicles, there were initially no indications that live non-invasive bacterial vectors could be used to induce immune responses against the foreign antigen that they produced. The novelty of the proposed research approach led to a number of basic questions that needed to be addressed.

- add food or "commensal" bacteria given at relatively high doses recognized as "self" or "non self" by the immune system of the host?.
- which kind of immune response (local/systemic, humoral /cellular, Th1/Th2, MHC-classI/-classII restricted) can be elicited by non-pathogenic non-invasive live vectors?.
- What is the immunomodulation capacity of different LAB?.
- Should a foreign antigen expressed by recombination LAB remain intracellular, be secreted or surface bound?.
- What level of antigen expression will be necessary to elicit a significant immune response?.
- Is bacterial colonization an advantage (long lasting immunity) or a disadvantage (induction of tolerance)?.
- How easily can a recombinant bacterium establish itself transiently in an equilibrated endogenous microflora?.
- What is the fate of LAB strains given locally? How stable are they *in vivo*?.

By nature, these points cover three interconnected areas: microbiology, molecular biology and immunology. The selection of model vaccine species or strains represents a critical step as the basic choice of using colonizing (transiently implanted in the normal microflora) versus non-colonizing (very short persistence in the host) isolates leads to quite different systems. In the first case, it is expected that a continuous *in vitro* synthesis of the antigen at the desired mucosal surface will trigger the underlying immune system. It might be speculated that a high level of synthesis will not be a prerequisite, especially when using immunostimulatory LAB strains. In the second case, the non-colonizing LAB may be considered as live microparticles that should be pre-loaded with the antigen and that will thus rely on high-level expression of protective antigens. Existing knowledge cannot predict if the optimal presentation of the antigen, a parameter which is known to affect its immunogenicity, will be identical or not in both systems. In the case of colonizers, strains appropriate for future human use have to be selected on the basic of safety, metabolic and physiology criteria. In addition, any of the potential vaccine strains should obviously be genetically amenable, in order to allow the expression of protective antigens or epitopes in different cellular locations (i.e. intracellularly, extracellularly or cell-surface exposed). Even though the genetics of LAB has progressed impressively during the last fifteen years, strain-specific optimization of expression, secretion and targeting vectors still require investigation. Concerning immunology, it is necessary to analyze the nature and the intensity of the immune response in relation to the mode of antigen presentation, the immunization route and the nature of the bacterial vector.

2.6.12 Safety

LAB are GRAS means Generally Regarded As Safe organisms. However, there are several case reports in the literature on systemic infections caused by LAB. The vast majority deals with severely immunocompromised patients. We have also to bear in mind that the taxonomy of several LAB has been reconstructed during the last decade, and the use of modern polyphasic taxonomy has reclassified several probiotic strains (Klein *et al*., 1998; Hoa *et al*., 2000; Temmermann *et al*., 2004). Nevertheless, there are a number of case reports of systemic spread of LAB, including strains of species used as probiotics. Using molecular biology techniques, however, there is probably only case where the causative agent, in this patient of a liver abscess, was indistinguishable from a strain used as probiotics, *L. Fhamnosus* GG (Rautio *et al*., 1999). Most patients report ingestion of raw milk or other dairy products, but not all (Pellizzer *et al*., 1996). Generally, probiotic strains carry a very risk of causing infection. Many probiotic products have been used traditionally over generations, and proven to be safe. Since different characteristics of strains listed above are strain specific, reports of systemic infections caused by various species should not make us exclude these species as probiotics. Each strain should be evaluated in tests for safety, but so far, there is no standard test(s) recommended.