STRAIN IDENTIFICATION, VIABILITY AND PROBIOTICS PROPERTIES OF *LACTOBACILLUS CASEI*

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ABSTRACT

The objectives of this study were: 1) to investigate differentiation of *Lactobacillus casei* group by carbohydrate fermentation, PCR, gene sequencing and pulsed field gel electrophoresis, 2) to investigate tolerance of *Lactobacillus* strains in the presence of acid, bile salts and the or bile salt deconjugation, 3) to investigate viability of freeze dried *Lactobacillus* strains at various temperature during storage and their proteolytic activity, 4) to investigate the growth, viability and activity of *Lactobacillus* strains in skim milk containing prebiotics and 5) to investigate in vitro inhibition of *Helicobacter pylori* by *Lactobacillus casei* strains.

Lactobacillus strains are a major part of the micorflora of the gut and of fermented dairy products, and are found in a variety of environments. *Lactobacillus casei, Lactobacillus paracasei, Lactobacillus rhamnosus* and *Lactobacillus zeae* form closely related taxonomic group within the facultatively heterofermentative lactobacilli. The classification and nomenclature of these bacteria are controversial. In this study, relationships between these species were investigated using type strains and dairy industry isolates examined with DNA-based techniques and conventional carbohydrate tests. Carbohydrates fermentation patterns gave poor discrimination of some species, but PCR using specific primers targeted to sequences of the 16S rRNA gene discriminated 4 types consistent with the currently recognized species. Pulsed-field agarose gel electrophoresis of chromosomal *Not* I restriction fragments identified 18 different band patterns from 21 independent *Lactobacillus* isolates and confirmed the identity of *L.casei* strains from 2 cultures collections (CSCC 5203 and ASCC 290), both representing the type strains of *L. casei*. Some isolates were reclassified as *L. rhamnosus* as a natural component of the microflora of dairy foods and dairy environments. These methods can provide a practical basis for discrimination of the species and identification of individual industrial strains.

Twenty two different *Lactobacillus* strains were screened for their acid and bile salt tolerance and for their bile salt deconjugation ability. All *Lactobacillus* strains were enumerated using MRS agar at 37^0C for 20 h. To evaluate effect of acid, MRS broth was adjusted to pH 3.0, 2.5, 2.0 and 1.5 with 5 N HCl. All strains were incubated for 3 h and aliquots were taken every hour up to 3 h and all strains were enumerated by pour plate method. ASCC 1520, ASCC 1521, ASCC 279, ASCC 290, ASCC 292, CSCC 2607 and ATCC 15820 had shown growth at pH 1.5 up to 1 h of incubation while same seven bacterial strains survived 3 h of incubation at pH 2.0. None of bacterial strains survived after 1 h of incubation at pH 1.5 while at pH 2.0 other than seven *Lactobacillus* strains had shown poor to moderate growth up to 3 h of incubation. All twenty two *Lactobacillus* strains had shown moderate to excellent growth at pH 2.5 and 3.0 up to 3 h of incubation.

To evaluate tolerance of bile salts, twenty two *Lactobacillus* strains were inoculated to MRS broth at pH 4.5 and filter sterilized bile salt concentrations of 1.0% and 1.5%. Aliquots were taken every hour up to 3 h and all samples were enumerated by pour plate method. All *Lactobacillus* strains had shown average to excellent growth at 1% bile concentration. CSCC 2607 showed highest growth at 3 h incubation followed by ATCC 15820 and ASCC1521. Seven *Lactobacillus* strains ASCC 1520, ASCC 1521, ASCC 279, ASCC 290, ASCC 292, CSCC 2607 and ATCC 15820 had shown growth up to 3 h of incubation at 1.5 % bile concentration while rest of the bacterial strains showed growth ranging between 1 h to 2 h.

Finally, Best seven *Lactobacillus* strains were screened for their deconjugation ability. Selected *Lactobacillus* strains were supplemented with sodium taurocholate, sodium glycocholate and combination of both respectively. ASCC 1521 had shown highest deconjugation ability with both glycine- and taurine- conjugated bile salts followed by ATCC 15820. CSCC 2607 had shown least conjugation ability. ASCC 1521 showed highest cholic

acid liberation while ASCC1520 showed lowest deconjugation ability against a mixture of glycine- and taurine- conjugated bile salts.

The viability and proteolytic activity of *Lactobacillus* strains at various temperatures during 12 month of storage was assessed. Seven *Lactobacillus* strains ASCC 1520, ASCC 1521, ASCC 279, ASCC 290, ASCC 292, CSCC 2607 and ATCC 15820 were freeze dried and stored at -18^{0}C, 4^{0}C and 20^{0}C for 12 months. *Lactobacillus* cell counts were enumerated using MRS agar at 37^{0}C for 72 h under aerobic conditions at 0, 1, 2, 4, 6, 9 and 12 mo. Freeze dried bacteria stored at 20^{0}C showed the greatest decline in the viability, while those stored at -18^{0}C showed least decrease. All freeze dried seven strains stored at -18^{0}C were also enumerated over night in MRS broth for proteolytic activity. Proteolytic activity was measured by OPA-based spectrophotometric assay at 0, 1, 2, 4, 6, 9 and 12 mo. CSCC 2607 showed highest proteolytic activity followed by ASCC 1520 and ASCC 279. In general all strains showed moderate to good proteolytic activity.

Seven *Lactobacillus* strains (ASCC 1520, ASCC 1521, ASCC 279, ASCC 290, ASCC 292, CSCC 2607 and ATCC 15820) were assessed for viability, doubling time, pH changes, and production of acetic and lactic acid in the presence of prebiotics during refrigerated storage. All seven strains were cultured anaerobically at 37^{0}C for 48 h in 12% (w/v) reconstituted skim milk containing 5% (w/v) Hi-maize^{TM}, lactulose, inulin or raftilose. Viability of all seven strains in the presence of Hi-maize^{TM}, lactulose, inulin or raftilose was determined. Viability of each *Lactobacillus* strains was assessed before and after 4 weeks of refrigerated storage at 4^{0}C. Mean doubling time (T_d) and specific activity (μ) in the presence of prebiotic was also measured. Concentrations of acetic and lactic acids produced by *Lactobacillus* strains during fermentation were determined. The doubling time (T_d) ranged from 301 to 751 min. The lowest doubling time was for CSCC 2607 followed by ASCC 279 with Hi-maize ^{TM}. Viability

of lactobacilli after four weeks was greatest with inulin. The average pH after 4 weeks of storage in skim milk was 4.34 (ASCC1520 with raftilose) to 4.10 (ATCC15820 with inulin). The highest concentration of acetic acid was produced by ATCC 15820 with 5% (w/v) lactulose, while the greatest concentration of lactic acid was produced by ATCC 15820 with 5% (w/v) Hi-maize ™. The growth, activity and retention of viability are specifically dependent on *Lactobacillus* strain studied. The addition of prebiotics to products containing probiotic bacteria such as *Lactobacillus* strains will have a significant effect, where unabsorbed prebiotics can be selectively utilised by them in the gut. Hence, a combination of a suitable *Lactobacillus* strain with a specific prebiotic would be a feasible approach in administering the beneficial bacteria *in vivo*.

Antagonistic effect of twenty two different *Lactobacillus* spp. were determined against one type strain of *Helicobacter pylori* ATCC 11637 and two patient's isolates Fiona 21174, U 3119288, respectively. Seven good *Lactobacillus* strains ASCC 1520, ASCC 1521, ASCC 279, ASCC 290, ASCC 292, CSCC 2607 and ATCC 15820 were selected for further assessment. The unadjusted and neutralised supernatants obtained from all seven *Lactobacillus* spp showed inhibition against all three *Helicobacter pylori* isolates. ASCC 1521, ASCC 290, ASCC 292 and ASCC 292 had shown better inhibition than rest of *Lactobacillus* spp. Co-culturing of ASCC 1521, ASCC 290, ASCC 292 and ASCC 292 with all three *Helicobacter pylori* showed high inhibition against this pathogen. All strains of *Lactobacillus* produced variable concentrations of acetic acid and lactic acid.

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LIST OF PUBLICATIONS

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2. Desai, A. R., Powell, I. B. and Shah, N. P. 2006. Discrimination of dairy industry isolates of the *Lactobacillus casei* group. *Journal of Dairy Science* 89:3345-3351.

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Conference abstracts

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2. Desai, A.R., Powell, I.B. and Shah, N.P. 2002 Differentiation of Lactobacilli: PCR, PFGE and Gene sequencing. Australian Society for Microbiology, Melbourne, September 29 - October 3, 2002

LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
CFU	Colony Forming Units
CSIRO	Commonwealth Scientific and Industrial Research Organisation
DNA	Deoxyribonucleic acid
DP	Degree of polymerisation
F6PPK	Fructose-6-phosphoketolase
FOS	Fructo-oligosaccharides
GIT	Gastrointestinal Tract
H.	*Helicobacter*
HPLC	High performance Liquid chromatography
L.	*Lactobacillus*
LAB	Lactic Acid Bacteria
MRS	De Mamm, Rogosa and Sharpe
PCR	Polymerase chain reaction.
PFGE	pulsed-field gel electrophoresis
NDO	Nondigestible oligosaccharide
rDNA	Ribosomal Deoxyribonucleic Acid
RSM	Reconstituted Skim Milk Powder
SD	Standard Deviation

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Introduction

Lactic acid bacteria including *Lactobacillus* spp. Such as *Lactobacillus casei, Lactobacillus acidophilus,* and *Bifidobacterium* spp. are becoming very popular in dairy industries due to their therapeutic benefits. Some health benefits include improvement in intestinal disorders and lactose intolerance, altered vitamin content of milk, antagonism against various pathogenic organisms and antimutagenic and anti-carcinogenic activities. These bacteria are widely used in the production of fermented foods and beverages and contribute both sensory qualities of the food and the prevention of spoilage. These organisms have added a new dimension to the importance of fermented milks in human nutrition and health. Moreover, they are present in large numbers in the normal human and animal gastrointestinal flora (Sgouras *et al.*, 2004). Health promoting benefits of consumption of LAB have been known for several years, since Metchnikoff (1908) first attributed longevity of Bulgarian peasants to consumption of fermented milks. The term ‘probiotic’ was first described by Fuller (1989) as ‘a live microbial feed supplement that beneficially affects the host by improving its intestinal microbial balance’. Schrenzenmeir and de Verse (2001) redefined probiotics to help broaden the scope, while emphasizing the fact that other compartments in the body, besides the intestines, might be targets for probiotic organisms. They defined probiotics as ‘a preparation or product containing viable, defined micro-organisms in sufficient numbers that alter the micorflora in a compartment of the host and by that exerts health effects in this host’. Recent research has credited several health benefits to probiotic organisms that are indigenous to the gastrointestinal tract, as well as those consumed through probiotic products. These include their ability to relieve symptoms of lactose

intolerance (DeVerse *et.al.,* 1992), increase immune function, cholesterol lowering potential (Noh *et al.,* 1997), antimutagenic activity (Lankaputhra and Shah, 1998) and treatment of diarrhea (Guandilini *et al.,* 2000) to name a few. The 'natural' target of ingested probiotics is the intestine, its microflora and the associated immune system, and therefore investigations and clinical studies of non-intestinal infections are rather scarce (de Verse and Schrenzenmeir, 2002). Therapeutic activity of probiotic bacteria can be due to competition with pathogens for nutrients and mucosal adherence, production of antimicrobial substances, and modulation of mucosal immune functions (O'sullivan *et al.,* 2005).

Reports also claim a correlation between anti-hypersensitivity and consumption of fermented milks containing *L. casei*. The human gastrointestinal tract constitutes a complex microbial ecosystem (Simon & Gorbach, 1986). More than 400 different species have been identified in feces of a single subject (Finegold *et al*., 1977; Moore *et al*., 1974). The equilibrium that exists in the large intestine is dynamic and is affected by age, diet composition and other environmental factors. Although these factors are significant, the continued maintaince of the intestinal microflora, which predominantly contains beneficial species such as probiotic bacteria, can improve our well being. The human gastrointestinal tract is known to possess active clearance mechanisms for microorganisms, and it has proven difficult to introduce new bacterial strains into this ecosystem (Savage, 1979). Constituents of the normal flora and some pathogenic bacteria possess the ability to colonise the mucosal surface of gastrointestinal tract and carries out a process of fermentation consuming substrates and producing end products that significantly influence our health (Haenel, 1961).

Some of the commonly known probiotics belong to the lactobacilli and bifidobacteria genus. Strains of *Lactobacillus casei, L. acidophilus*, *Bifidobacterium bifidum, B. longum* and *B.*

infantis have been identified as possessing probiotic properties, and many have been used to treat gastrointestinal diseases. They have shown to exert either bacteriostatic or bactericidal activity against several pathogens. Many probiotics are now widely marketed through food products such as yoghurt, fermented milks, fermented juices and freeze dried supplements.

The concept of the group name 'lactic acid bacteria' was created for bacteria causing fermentation and coagulation in milk, and defined as those which produce lactic acid from lactose. The family name Lactobacteriaceae was applied by Orla-Jensen (1919) to a physiological group of Gram-positive rods and cocci. Today, same physiological group is now subdivided into four genera *Streptococcus*, *Leuconostoc*, *Pediococcus*, and *Lactobacillus*. Members of *Lactobacillus casei* are Gram-positive, facultatively anaerobic, catalase-negative, facultatively heterofermentavie, non-spore-forming rods and are isolated from many habitats (e.g., meats, milks, dairy products, sour dough, silage, and sewage). Strains of *Lactobacillus* are important for many food fermentations and are normal constituents of intestinal microflora. Some *Lactobacillus* strains have desirable and functional characteristics (Saxelin *et al.,* 1996). The *Lactobacillus casei* group contains a number of well-known probiotics strains including *L. casei* shirota (Sugita & Togawa, 1994) and *L. rhamnosus* GG (Saxelin, 1997). The identification of *Lactobacillus* strains within this group (*L. casei, L. paracasei, L. rhamnosus* and *L. zeae*) is important for both basic studies and applications in food industries. Most of these strains have very similar physiological properties and nutritional requirements and grow under similar environmental conditions. Traditional phenotypical tests for *Lactobacillus* identification can be difficult to interpret. The techniques are also time consuming and results are often ambiguous (Charteris *et al*., 1997; Hammes *et al*., 1992). While this group of *Lactobacillus* can be readily distinguished from other members of the *Lactobacillus* genus by fermentation profiles (Hammes

et al., 1992), it is not possible to unequivocally distinguish between these four species on the basis of fermentation patterns. They form a closely related taxonomic group. In recent years, the taxonomy of this group has changed considerably with increasing knowledge of genomic structure and phylogenic relationships between *Lactobacillus* species (Klein *et al*., 1998; Tisala-Timisjarvi *et al*., 1999). As commercially available probiotic strains are being produced and marketed for human consumption on a large scale, it is important to identify them to species and strain levels.

The bacterial populations of the human gastrointestinal tract constitute a complex ecosystem (Simon & Gorbach, 1986). More than 400 bacterial species have been identified in feces of a single subject (Finegold *et al*., 1977). Gastrointestinal tract is known to possess active clearance mechanisms for microorganisms, and it has proven difficult to introduce new bacterial strains into this ecosystem (Paul & Hoskins, 1972). There is, however, host specificity in colonization by individual species; for example, *Lactobacillus acidophilus, L. fermentum* and *L. plantarum* are commonly found in the feces of humans; whereas *L. delb.* spp. *bulgaricus,* the organism used in combination with *Streptococcus thermophilus* to make yoghurt, is unable to colonise the bowl and is not isolated in the feces (Finegold *et al*., 1977). In a number of different circumstances it can be beneficial to alter the intestinal microflora by introducing lactobacilli. The nutritional and therapeutic benefits derived from this approach have been discussed in the review article by Gorbach (1990). The claimed benefits of bacterial supplementation include increased nutrient utilization, alleviation of lactose intolerance, treatment of hepatic encephalopathy and intestinal infections, and inhibition of bacterially derived generation of carcinogens in the intestinal tract (Finegold *et al*., 1977).

Metchnikoff (1908) was the first to establish that microorganisms were responsible for the beneficial effects associated with lactobacilli fermented foods. He attributed the longevity of the Bulgarians to consumption of milk products fermented with *Lactobacillus bulgaricus*. Lactic acid bacteria have been widely used in making variety of cultured dairy foods for desirable flavor or other characteristic, and they have been known to produce the lactic acid and others, which influence growth or survival of other bacteria as well as themselves (Kim *et al*., 1980). The harmful effects of the undesired bacteria can be overcome by establishing a new balance between intestinal flora, through ingestion of lactic organisms present in cultured dairy products. Bacterial modification of primary bile acids is catabolism of bile acids by microorganisms and deconjugation of conjugated bile acids (Aries & Hill, 1970). There is scant information available about survival of *L. casei* in acidic conditions and varying concentrations of bile and bile are normally encountered in the gastrointestinal tract and their deconjugation ability.

Fuller (1989) has redefined a probiotic as ‘a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance’. A prebiotic is a nondigestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health (Gibson & Roberfroid, 1995). The majority of simple sugars and oligosaccharides ingested and digested by humans are absorbed in the small intestine (Bond *et al*., 1980). However some prebiotic such as lactose, raffinose, stachyose and fructooligosaccharides (such as oligofructose or inulin) are able to reach the colon intact (Roberfroid *et al*., 1993). Prebiotics have been selectively manufactured to contain several or all of the following attributes; active at low dosages, varying viscosity, lack of side effects, varying sweetness, control of microflora modulation, persistence through the colon, good storage and processing stability and inhibition of

pathogen adhesion. Established and possible effects of prebiotics include nondigestibility and low energy value, stool bulking effect and modulation of the gut flora, promotion of bifidobacteria and repression of clostridia. Oligosaccharides are mostly a group of short chain non-digestible polysaccharides that occur naturally in foods. They are typically defined as glycosides that contain between 3 to 10 sugar moieties and are characterized by the type and sequence of the monosaccharide moieties present (Crittenden, 1999). In the last 10 years, there has been an increasing interest in the consumption of probiotics and functional foods in Western diets (O'sullivan, 1996). Probiotic bacteria are able to suppress potentially pathogenic microorganism in the gastrointestinal tract and enhance the population of beneficial microorganisms (Yaeshima *et al*., 1997).

The health benefits derived by the consumption of foods containing probiotic bacteria are well documented and more than 90 probiotic products are available worldwide (Shah, 2000). To provide health benefits, the suggested concentration for probiotic bacteria is 10^6 cfu/g of a product (Shah, 2000). However, studies have shown low viability of probiotics in market preparations (Shah *et al*., 1995). A number of factors have been claimed to affect the viability of probiotic bacteria in fermented food including acid and hydrogen peroxide produced by bacteria, oxygen content in the product, and oxygen permeation through the package (Shah, 2000). Viability of probiotic bacteria in fermented products declines over time because of the acidity of the product, storage temperature, storage time, and depletion of nutrients (Dave & Shah, 1997a). Loss of viability of probiotic bacteria occurs in fermented products, and these products have limited shelf life (Dave & Shah, 1996). However, in order to achieve maximum viability in a product, in the gut and maximum health benefits, there is a need to have a better understanding of this organism as an emerging probiotic.

Proteolysis in milk and dairy products has both beneficial and undesirable effects in the dairy industry. The process of cheese ripening results from activities of natural milk proteinase, rennet, and proteinases of the starter culture (Thomas & Mills, 1981). *Lactobacillus casei* is recognized to be an important component of the micorflora in several kinds of hard and semi-hard cheeses, predominating over other mesophilic lactobacilli (Taylor & Sharpe, 1958). Robertson (1964) examined the flora of cheddar cheese from several countries and found *L. casei* to be the most frequently occurring species in each case.

Helicobacter pylori is a spiral-shaped, Gram-negative rod that has developed sophisticated strategies to colonise epithelial cells lining the antrum of the stomach and to survive in acidic environments. Bacterial interference between normal flora and pathogenic organisms has been described since the late 19th century (Reid *et al*., 1990). Recently, attention has been paid to the interactions between *H. pylori* and probiotic lactobacilli. *H. pylori* colonization was inhibited in *L. salivarius* fed gnotobiotic BALB/dc mice, and *H. pylori* antibody titers became marginal, while *H. pylori* colonized in large numbers and caused active gastritis in *Lactobacillus*-free mice (Kabir *et al*., 1997). In another study, probiotics including *L. acidophilus* has been reported to have antagonistic action against ulcer causing bacterium, *H. pylori* (Lorca *et al*., 2001). Cocconier *et al*. (1998) have reported the oral administration of a spent culture supernatant of *L. acidophilus* resulted in the suppression of *H. felis* in a maurine infection model. In addition, an *L. acidophilus* culture supernatant was effective in vitro and had a partial, long-term suppressive effect on *H. pylori* in humans (Michetti *et al*., 1999). There is substantial evidence that the intestinal ecoflora can protect against infections and that the disruption of this microbial balance can in increase susceptibility to infections. Many in vitro and in vivo studies

have shown that the normal intestinal flora is an extremely effective barrier against pathogenic and opportunistic microorganisms (Fuller, 1991).

This study was focused in three different areas. First part of the study aimed to carry out DNA fingerprinting of *Lactobacillus casei* species to make sure that the strains are properly speciated before using this project. Second part of this project was focused to examine some functional characteristics of *Lactobacillus* spp. including tolerance against acid, bile and their bile deconjugation ability, Survival freeze-dried *L. casei* strains and their and proteolytic activity. Third and finally the study was focused on inhibition properties of *H. pylori* by *L. casei.*

The main aims of this project were to:

1. To genetically characterize strains of *Lactobacillus* spp. and select the best strain based on survival under adverse conditions, proteolytic activity, and
2. Effects against *H. pylori*.

The specific objectives of this study were:

1. To investigate differentiation of *Lactobacillus casei* group by carbohydrate fermentation, PCR, gene sequencing and pulsed field gel electrophoresis,
2. To investigate tolerance of *Lactobacillus* strains in the presence of acid, bile salts and deconjugation of bile salts,
3. To investigate viability and proteolytic activity of freeze dried *Lactobacillus* strains at various temperature during storage,
4. To investigate the growth, viability and activity of *Lactobacillus* spp in skim milk containing prebiotics,
5. To investigate inhibition of *Helicobacter pylori* by *Lactobacillus* spp. i*n vitro*.

1.0 LITERATURE REVIEW

1.1 Lactic acid bacteria

1.1.1. Historical background of lactic acid bacteria

Lactic acid- producing fermentation is an old invention. Many different cultures in various parts of the world have used fermentation to improve the storage qualities and nutritive value of perishable foods such as milk, vegetables, meat fish and cereals. The organisms that produce this type of fermentation, lactic acid bacteria, have had an important role in preserving foods. In developed world, lactic acid bacteria are mainly associated with fermented dairy products such as cheese, buttermilk, and yogurt. The use of dairy starter cultures has become an industry during this century.

The concept of the group name 'lactic acid bacteria' was created for bacteria causing fermentation and coagulation of milk, and defines as those which produce lactic acid from lactose. The family name Lactobacteriaceae was applied by Orla-Jensen (1919) to a physiological group of bacteria producing lactic acid alone or acetic and lactic acids, alcohol and carbon dioxide. Today, lactic acid bacteria are regarded as synonymous by and large with the family Lactobacteriaceae (Breed *et al*., 1957).

Since the days of Russian scientist Metchnikoff, lactic acid bacteria have also been associated with beneficial health effects. Today, an increasing number of health food and so-called functional foods as well as pharmaceutical preparation are promoted with health claims based on the characteristics of certain strains of lactic acid bacteria. Most of these strains, however, have not been thoroughly studied, and consequently the claims are not well substantiated. Moreover, health benefits are judged mainly using subjective criteria. Additionally,

the specific bacterial strains used in the studies are often poorly identified. Most information about the health effects of lactic acid bacteria is thus anecdotal. There is clear need for critical study of the effect on health of strain selection and the quality of fermented foods and their ingredients.

Lactic acid bacteria are a group of Gram-positive bacteria united by a constellation of morphological, metabolic, and physiological characteristics. They are non-sporing, carbohydrate-fermenting lactic acid producers, acid tolerant of non-aerobic habitat and catalase negative. Typically they are non-motile and do not reduce nitrite. They are subdivided into four genera *Streptococcus, Leuconstoc, Pediococcus,* and *Lactobacillus*. Recent taxonomic revisions suggest that lactic acid bacteria group could be comprised of genera *Aerococcus, Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Pediococcus, Streptococcus, Tetragenococcus,* and *Vagococcus*. Originally, bifidobacteria were included in the genus *Lactobacillus* and the organism was referred to as *Lactobacillus bifidus*.

Although the classification of lactic acid bacteria into different genera is mainly based on the characteristics used by Orla-Jensen (1919); however, confusion was still prevalent when the monograph of Orla-Jensen (1919) appeared. This work has had a large impact on the systematic of lactic acid bacteria, and, although revised to some extent, it is still valid and the basis of classification remarkably unchanged. The classification of lactic acid bacteria into different genera is largely based on morphology, mode of glucose fermentation, growth at different temperatures, and configuration of the lactic acid produced, ability to grow at high salt concentrations, and acid or alkaline tolerance. Even some of the newly described genera of lactic acid bacteria, additional characteristics such as fatty acid composition and motility are used as the basis of classification.

The term lactic acid bacteria was used synonymously with “milk souring organisms.” Important progress in the classification of these bacteria was made when the similarity between milk-souring bacteria and other lactic-acid producing bacteria of other habitats was recognized (Axelsson, 1993). lactic acid bacteria 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 mouth, intestine, and vagina of mammals. The genera that, in most respects, fit the general description of the typical lactic acid bacteria are (as they appear in the latest *Bergey’s Manual* from 1986) *Aerococcus*, *Lactobacillus*, *Leuconostoc*, *Pediococcus*, and *Streptococcus*. The genera *Lactobacillus*, *Leuconostoc*, and *Pediococcus* have largely remained unchanged, but some rod-shaped lactic acid bacteria, previously included in *Lactobacillus*, is now forming the genus *Carnobacterium* (Collins *et al*., 1987).

1.1.2. Classification at genus level

The basis for the classification of lactic acid bacteria in different genera has essentially remained unchanged since the work of Orla-Jensen (1919). Although their 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 lactic acid bacteria genera. Thus lactic acid bacteria can be divided into rods (*Lactobacillus* and *Carnobacterium*) and cocci (all other genera).

An important characteristic used in the differentiation of the lactic acid bacteria 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, lactic acid bacteria can be divided into two groups: homofermentative, which convert glucose almost quantitatively to lactic acid, and heterofermentative, which ferment glucose to lactic acid, ethanol/acetic acid, and CO_2 (Sharpe,

1979). In practice, a test for gas production from glucose will distinguish between the groups (Sharpe, 1979). More detailed glucose metabolic pathways are shown in Fig. 1. Leuconostocs and a subgroup of *Lactobacillus* are heterofermentative; all other lactic acid bacteria are homofermentative.

Growth at certain temperatures is mainly used to distinguish between some of the cocci. Enterococci grow at both 10^0C and 45^0C, lactococci and vagococci at 10^0C, but not at 45^0C. Streptococci do not grow at 10^0C, while growth at 45^0C in dependent on the species (Axelsson, 1993). 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 genus *Tetragenococcus*. Tolerances to acid and/or alkaline conditions are also useful characteristics.

Enterococci are characterised 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 Leuconostoc and most heterofermentative lactobacilli, as the former produce only D-lactic acid and the latter a racemate (DL-lactic acid). A summary of the differentiation of the lactic acid bacteria genera with classical phenotypic tests is shown in Table 1.1.

Table 1.1. Differential characteristics of lactic acid bacteria

Characteristic	Rods			Cocci					
	Carnob.	*Lactob.*	*Aeroc.*	*Entero*	*Lactoc. Vagoc.*	*Leucon.*	*Pedioc.*	*Streptoc.*	*Tetragenoc.*
Tetrad formation	-	-	+	-	-	-	+	-	+
CO_2 from glucose [b]	-	±	-	-	-	+	-	-	-
Growth at 10^0 C	+	±	+	+	+	+	±	-	+
Growth at 45^0 C	-	±	-	+	-	-	±	±	-
Growth in 6.5% NaCl	ND[d]	±	+	+	-	±	±	-	+
Growth in 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

[a] +, positive, -, negative, ±, response varies between species; ND, not detected.
[b] Test for homo- or hetrofermentation 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 species.

1.1.3 Metabolism of lactic acid bacteria

The essential feature of lactic acid bacteria metabolism is efficient carbohydrate fermentation coupled to substrate-level phosphorylation. The generated ATP is subsequently used for biosynthesis purposes. lactic acid bacteria as a group exhibit an enormous capacity to degrade different carbohydrates and elated compounds. Generally, the predominant end product is of course, lactic acid (> 50% of sugar carbon). It is clear however, that lactic acid bacteria adapt to various conditions and change their metabolism accordingly. This may lead to significantly different end-product patterns. This section will describe the well-known fermentation pathways and how various sugars are fermented. It is also attempting to show some of the more unusual features of lactic acid bacteria metabolism, which may be of importance in their natural habitat.

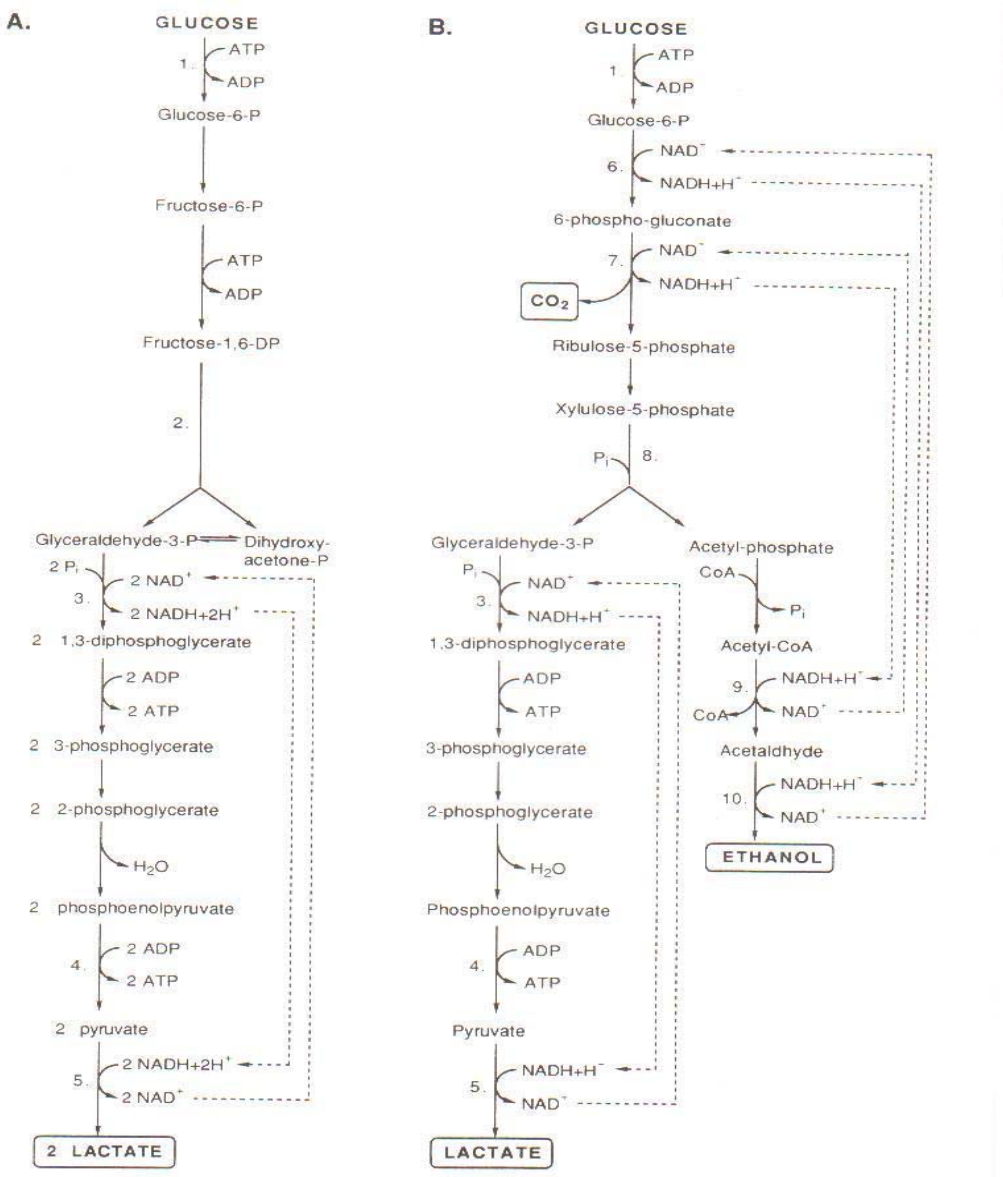

Figure1.1 Major fermentation pathways of glucose (A) Homolactic fermentation (glycolysis, Embden-Meyerhof pathway); (B) Heterolactic fermentation (6-phosphogluconate/phosphoketolase pathway). Selected enzymes are numbered: 1. Glucokinase; 2. Fructose-1,6-diphosphate aldolase; 3. Glyceraldehyde-3-phosphate dehydrogenase; 4. Pyruvate kinase; 5. Lactate dehydrogenase; 6. Glucose-6-phosphate dehydrogenase; 7. 6-Phosphogluconatedehydrogenase; 8. Phosphoketolase; 9. Acetaldehyde dehydrogenase; 10. Alcohol dehydrogenase. (Axelsson, 1993)

1.1.3.1 Hexose fermentation

There are two major pathways for hexose (e.g., glucose) fermentation occurring within lactic acid bacteria. Pathways are shown in Figure1.1. The transport and phosphorylation of glucose may occur as outlined in the Figure. Glycolysis (Embden-Meyerhof pathway), used by all lactic acid bacteria except leuconostocs and group C lactobacilli, is characterized by the formation of fructose-1-6-diphosphate (FDP), which is split by an FDP aldolase into dehydroxyacetonephosphate (DHAP) and glyceraldehydes-3-phosphate (GAP). GAP (and DHAP via GAP) is then converted to pyruvate in a metabolic sequence including substrate level phosphorylation at two sites. Under normal conditions, i.e., excess sugar and limited access to oxygen; pyruvate is reduced to lactic acid by a NAD^+ -dependent lactate dehydrogenase (nLDH), thereby reoxidizing the NADH formed during the earlier glycolytic steps. A redox balance is thus obtained, lactic acid is virtually the only end product, and the metabolism is referred to as a homolactic fermentation.

The other main fermentation pathway has had several designations, such as the pentose phosphate pathway, the pentose phosphoketolase pathway, the hexose monophosphate shunt, and as used by Kandler and Weiss (1986) in *Bergey's Manual*, the 6-phosphogluconatepathway. It is characterised by initial dehydrogenation steps with the formation of 6-phosphogloconate, followed by decarboxylation. The remaining pentose-5-phosphate is split by phosphoketolase into GAP and acetyl phosphate. GAP is metabolized in the same way as for the glycolytic pathway, resulting in lactic acid formation. When no additional electron acceptor is available, acetyl phosphate is reduced to ethanol via acetyl CoA and acetaldehyde. Since this metabolism leads to significant amounts of other end products (CO_2, ethanol) in addition to lactic acid, it is referred to as a heterolactic fermentation. Both fermentation pathways are shown in Figure 1.1.

1.2. History of *Lactobacillus*

1.2.1 Historical background of Lactobacillus

The genus *Lactobacillus* is by far the largest of the genera included in lactic acid bacteria. 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 Stackebrandt, 1983). The heterogeneity and the large number of species are due to definition of the genus, which essentially are rod-shaped lactic acid bacteria. Such a definition is comparable to an arrangement where the entire coccoid lactic acid bacteria were included in one genus.

Table 1.2. Arrangement of the genus *Lactobacillus* (Kandler and Weiss, 1986).

Characteristic	Group I: Obligately Homofermentative	Group II: Facultatively Heterofermentative	Group III: Obligately Heterofermentative
Pantose fermentation	-	+	+
CO_2 from glucose	-	-	+
CO_2 from gluconate	-	+[a]	+[a]
FDP aldolase present	+	+	-
Phosphoketolase present	-	+[b]	+
	L. acidophilus	*L. casei*	*L. brevis*
	L. delbruckii	*L. curvatus*	*L. buchneri*
	L. helveticus	*L. plantarum*	*L. fermentum*
	L. salivarius	*L. sake*	*L. reuteri*

[a] When fermented
[b] Inducible by pentoses.

Table 1.2 summarizes the characters used to distinguish among the three groups and some of the more well-known species included in each group. The physiological basis for the division is (generally) the presence or absence of the key enzymes of homo-and heterofermentative sugar metabolism, fructose-1, 6-diphosphate aldolase, and phosphoketolase, respectively (Kandler,

1983, 1984; Kandler and Weiss, 1986). Further details regarding the division of lactic acid bacteria in homo-and heterofermentative please refer (Figure 1.1).

Lactobacilli are wide spread in nature, and many species have found applications in the food industry. They are Gram-positive, non-spore-forming, rods or coccobacilli with a G + C content of DNA usually <50mol%. Lactic acid bacteria due to their higher molecular percentage of G + C contents in DNA and are placed in the Actinomycete branch as presented Table 1.4, while other lactic acid bacteria are placed in the *Clostridium* branch. Presented in Tables 1.3 and 1.4 compares the G + C contents in DNA of several genus of lactic acid bacteria and phylogenetic relationship of lactic acid bacteria according to G + C mol percent content in DNA, respectively.

Table 1.3. DNA base composition of different lactic acid bacteria (Salminen and von Wright, 1993).

Genus	G + C % range
Lactobacillus	34.7-50.8
Streptococcus	33-44
Leuconstoc	39-42
Bifidobacterium	57.2-64.5

Table 1.4. Phylogenetic relationship of lactic acid bacteria based on the mol percent of G + C content in DNA (Salminen and von Wright, 1998)

Mol % of G + C content in DNA	Branch	
	Clostridium	Actinomycete
	<50	> 50
	Lactobacillus	*Bifidobacterium*
	Lactococcus	*Propionibacterium*
	Enterococcus	*Microbacterium*
	Leuconostoc	*Corynebacterium*
	Pediococcus	*Brevibacterium*
	Streptococcus	*Atophobium*
	Staphylococcus aureus	
	Bacillus subtillis	

They are strictly fermentative, aero-tolerant or anaerobic, aciduric or acidophilic and have complex nutritional requirements (e.g. for carbohydrates, amino acids, peptides, fatty acid esters, salts, nucleic acid derivatives, and vitamins). They do not synthesize porphyrinoids and thus, are devoid of hemedependent activities.

Lactobacilli are found in where rich, carbohydrates-containing substrate are available, and thus, in a variety of habitats such as mucosal membranes of humans and animals, (mainly in oral cavity, intestine, and vagina) and on plant material and fermenting food (Hammes *et al.,* 1991; Pot *et al.,* 1994). Lactobacilli are strictly fermentative, aero-tolerant to anaerobic, aciduric or acidophilic and they have complex nutritional requirements.

1.2.2 Grouping of Lactobacillus

The primary interest of Orla-Jensen's (1919) early description of the lactic acid bacteria was directed to identify these bacteria useful in the dairy industry, with the particular interest in the study of those bacteria occurring in Danish 'dairy cheese'. Orla-Jensen recognized 10 species

in his time. This number increased only slowly to 15 and 25 species, in the 7th and 8th editions of Bergey's Manual respectively. Finally 44 species have been recognized in the latest 9th edition of Bergey's Manual. The numbers of species are still increasing due to emerging of new taxonomic methods, which allow a more precise identification of strains isolated some time ago and, to some extent, from the continued investigation of habitats.

The latest grouping of lactobacilli by Kandler and Weiss (1986) relies on biochemical-physiological criteria and neglects classical criteria of Orla-Jensen such as morphology and growth temperature since many of recently described species did not fit into the traditional classification scheme. Unfortunately, the description of new species usually does not include the analysis of the end products derived from the fermentation of pentoses, and therefore, the enzymes of the pentose phosphate pathway may be present permitting a homofermentative metabolism of pentose in lactobacilli. Nevertheless, maintaining the traditional terms is justified with regards to hexose utilization. However, at low substrate concentration and under strictly anaerobic conditions, some facultatively heterofermentative species may produce acetate, ethanol and formate instead of lactate from pyruvate. Thus, the definitions have to be used in awareness of their limitations.

When glucose is used as a carbon source, lactobacilli could be homofermentative or heterofermentative. When homofermentative, they could produce more than 85% lactic acid, whereas the heterofermentative strains produce lactic acid, carbon dioxide, ethanol or acetic acid. In the presence of oxygen or other oxidants, increased amounts of acetate may be produced at the expense of lactate or ethanol. A total of 56 species of lactobacilli have been divided into three metabolic groups (Hammes and Vogel, 1995).

- **Group A**: *Obligatory homofermentative lactobacilli*: Hexoses are fermented to lactic acid by EMP pathway. Pentose or gluconate are not fermented.

- **Group B**: *Facultatively heterofermentavie lactobacilli*: Hexoses are fermented to lactic acid by EMP pathway. The organisms possess both aldolase and phosphoketolase and therefore, not only ferment hexose but alsopentoses (and often gluconate). In the presence of glucose, the enzymes of the phosphogluconate pathway and may be fermented.
- **Group C**: *Obligatory heterofermentavie lactobacilli*: the phospogluconate pathway ferments hexoses, yielding lactate, acetic acid (ethanol) and CO_2 in equimolar amounts. Pentose enters in this pathway and may be fermented.

Within these three groups the species are arranged according to their phylogenetic relationship. The letter indicates the affiliation of the *L. delbrueckii* group, b to the *L. casei-Pediococcus* group. Thus, the combination of the letter Aa defines a species as belonging to the obligatory homofermentative lactobacilli affiliated in the *L. delbrueckii* group, whereas Cb means that the species is obligatory heterofermentative phylogenetically belonging to the *L. casei-Pediococcus* group, etc.

The *L. casei* species belonging to the group facultatively a heterofermentavie organism comes under group B. Two species, *L. acetotolerans* and *L. hamsteri* constitute group Ba, meaning that phylogenetically these organisms fall into the *L. delbrueckii* group. The presence of the Lys-Dasp type peptidoglycan is consistent with this grouping.

Group Bb contains 15 species, 12 of which contain Lys-Dasp and three DAP in their peptidoglycan. In contrast to Kandler and Weiss (1986), Hammes and Vogel (1995) have included into group Bb *L. bifermentans* since, in agreement with the group definition; this organism possesses key enzymes, aldolase and phosphoketolase. *L. bifermentans* is characterized by fermenting glucose homofermentatively. However, dependent on the pH, lactate can be metabolized to ethanol, acetic acid and CO_2 and H_2. The utilization of lactate (and/or pyruvate) is rather common for group Bb-organisms.

It can be foreseen that changes will occur for the species *L. casei* and *L. paracasei*. With ample evidence, Dellaglio et al. (1991) presented a request for an opinion. They showed that the type strain of *L. casei* (ATCC 393) is not genetically closely related to several subspecies of *L. casei* as they were described by Kandler and Weiss (1986). This had led Collins *et al.* (1989) to describe *L. paracasei* sp. Nov. that included these types of strains. It appears, however, that the species *L. paracasei* should be rejected and changed for *L. casei*. The type strain of *L. casei* would have to be allotted to a new species that includes also one strain of *L. rhamnosus* and *L. zeae* (Kuznestov, 1959).

1.2.3 Description of the species

All cells are Gram-positive and non-spore-forming, usually catalase-negative, non-motile, and facultatively anaerobic unless otherwise stated. The grouping of the species together with the patterns of sugar fermentation and important physiological properties are given in Table 1.1. The numbering of species is in accordance with that used in these tables. The numbers given after the reference are the page numbers on which the descriptions of the species are provided. The thesis that were indicated in this are given below.

1.2.3.1. Lactobacillus casei (Orla-Jensen, 1919; Hansen and Lessel, 1971, P. 71) Group Bb. Cells are rods of 0.7 – 1.1 by 2.0 – 4.0 µm, often with square ends and tending to form chains. Riboflavin, folic acid, calcium pantothenate and niacin are required for growth. Pyridoxal or pyridoxamine is essential or stimulatory. Thiamine, vitamin B_{12} and thymidine are not required. Strains were isolated from milk and cheese, dairy products and dairy environments, sourdough, cow dung, silage, human intestinal tract, mouth and vagina, sewage. The type strain is ATCC 393.

1.2.3.2. *Lactobacillus paracasei* (Collins *et al*., 1989, p. 108) Group Bb. Cells are rod shaped, 0.8-1.0 by 2.0-4.0 µm, often with square ends, and occur singly or in chains, Growth at 10 and 40^0C. Some strains grow at 5 and 45^0C. A few strains (formerly *Lb. casei* subsp. *pseudoplantarum*) produce inactive lactic acid due to the activity of L-lactic acid racemase. Two subspecies are validly published.

1.2.3.2a Lactobacillus paracasei* subsp. *paracasei (Collins *et al*., 1989, p. 108) Strains were isolated from dairy products, sewage, silage, humans, and clinical sources. The type strain is NCDO 151 (Collins *et al*., 1989)

1.2.3.2*b *Lactobacillus paracasei* subsp. *tolerans (Collins *et al*., 1989, p. 108) tolerans tolerating, enduring; means survival during pasteurization of milk. Group Bb. Cells are rod shaped, 0.8-1.0 by 2.0-4.0 µm, often with square ends, and occur singly or in chains. Survives heating at 72^0C for 40 s. The strains are isolated from dairy products. The type strain is ATCC 25599 (Collins *et al*., 1989).

1.2.3.3. Lactobacillus rhamnosus (Collins *et al*., 1989, p. 108) (*Lactobacillus casei* subsp. *rhamnosus* Hansen, 1968, p. 76). Group Bb. Cells are rod shaped, 0.8-1.0 by 2.0-4.0 µm, often with square ends, and occur singly or in chains. Some strains grow at 48^0C. The strains were isolated from dairy products, sewage, humans, and clinical sources. The type strain is ATCC 7469 (Collins *et al*., 1989).

1.3. Taxonomic diversity of *Lactobacillus*

The human gastrointestinal tract contains hundreds of different bacterial species. (Tannock, 1995). Members of the genus *Lactobacillus* are commonly present as members of microbial communities and have received considerable attention with respect to their putative health conferring properties as probiotics (Goldin and Gorbach, 1992). *Lactobacillus* has worldwide industrial use as starters in the manufacturing of fermented milk products. Moreover, some of *Lactobacillus* strains have probiotic characteristics and are therefore included in fresh fermented products or used in capsular health products, such as freeze-dried powder. The use of some *Lactobacillus* strains as probiotics is based on studies that show that these species belong to the normal intestinal flora and that the strains have beneficial effects on human and animal health (Salminen *et al*., 1996).

Major bacterial species isolated from human gastrointestinal tract fall generally into three distinct categories. These include 1) organisms almost always present in large number, and constituting the indigenous and resident flora, e.g. *Bacteroides, Bifidobacterium*; 2) organisms normally present in small or moderate numbers, and part of the resident flora, e.g. Enterobacteriaceae, *Streptococcus* and *Lactobacillus*; and 3) organisms present in small numbers, probably contaminants from other regions of the body e.g. *Staphylococcus*, *Haemophilus*, etc., or from the environment, e.g. *Bacillus, Corynebacterium*, which constitute transient flora.

More specifically, organisms of the human gastrointestinal tract include diverse bacterial genera or families, and are divided into the following three groups: 1) Lactic acid bacteria in a broad sense, including *Bifidobacterium, Lactobacillus,* and *Streptococcus* (including *Enterococcus*); 2) Anaerobic group, including *Bacteriodaceae, Eubacterium, Peptococcaceae, Veillonella, Megasphera, Hemmiger, Clostridium* and *Treponema*; and 3) Aerobic group,

including *Enterobacteriaceae*, *Staphylococcus, Bacillus, Corynebacterium, Pseudomonas* and yeasts.

1.3.1. Species-specific identification

Traditionally, the identification of *Lactobacillus* has been based mainly on fermentation of carbohydrates, morphology, and Gram staining, and these methods are still used. The identification of *Lactobacillus* isolates by phenotypic methods is difficult because it requires, in several cases, determination of bacterial properties beyond those of the common fermentation tests (for example, cell wall analysis and electrophoretic mobility of lactate dehydrogenase) (Kandler and Weiss, 1986). In general about 17 phenotypic tests are required to identify a *Lactobacillus* isolate accurately to the species level (Hammes and Vogel, 1995). In recent years, the taxonomy has changed considerably with the increasing knowledge of genomic structure and phylogenetic relationships between *Lactobacillus* spp. (Klein *et al.,* 1998).

During the past 20 years, significant advances have been made in bacterial taxonomy of indigenous intestinal bacteria. Newly developed research methods such as DNA-DNA homology, rRDNA-DNA homology or the guanine-plus- cytosine (G+C) content of DNA have contributed much to the advances in bacterial taxonomy, and numerous new taxa of intestinal anaerobes were described. Results of DNA homology are used to indicate relationship among strains, establish genospecies, and enable selection of those phenotypic tests that are the most useful for reliable identification of new isolates. Many different types of bacteria, representing most bacterial groups, have been isolated from the intestine. Between 30-40 species constitute approximately 90% of the flora, but in order to be sure of identifying these species some hundred isolates from each sample should be examined. Such bacteria are generally identified on the base of their morphology as determined by Gram stain, fermentation reactions and metabolic tests. While several schemes have been developed for the identification of indigenous anaerobic bacteria, it is

still difficult to identify many of these organisms by conventional tests at species level. Differentiation of major intestinal bacterial groups according to Gram-staining, aerobic growth, spore production and major fermentation products are presented below in Table 1.5.

Table 1.5. Differentiation of major intestinal bacterial groups (Mitsuoka, 1992).

Bacterial group	Gram-staining	Aerobic growth	Spore production	Major fermentation products
Lactic acid bacteria group				
Lactobacillus	+	+	-	Lactic acid
Bifidobacterium	+	-	-	Acetic acid+ lactic acid
Streptococcus	+	+	-	Lactic acid
Anaerobic group				
Bacteroidaceae	-	-	-	Various products
Anaerobic curved rods	-	-	-	Succinic acid, butyric acid
Eubacterium	+	-	-	Various products
Peptococcaceae	+	-	-	Various products
Veillonella	-	-	-	Acetic acid + propionic acid
Megasphaera	-	-	-	Caproic acid + butyric acid
Gemmiger	-	-	-	
Clostridium	+/-	-	+	Various products
Treponema	-	-	-	
Aerobic group				
Enterobacteriaceae	-	+	-	
Staphylococcus	+	+	-	
Bacillus	+	+	+	
Corynebacterium	+	+	-	
Pseudomonas	-	+	-	
Yeasts	+	+	-	

The *Lactobacillus* commonly includes *Lactobacillus* casei and the taxonomically related species *L. paracasei* and *L. rhamnosus.* While this group of lactobacilli can be readily distinguished from other members of the *Lactobacillus* genus by fermentation profiles (Hammes *et al*., 1992), it is not possible to unequivocally distinguish between these three species on this basis. The identification of *L. casei* by polymerase chain reaction is important for basic studies and applications in food industries. These lactobacilli commonly include *L. casei* and the taxonomically related species *L. paracasei* and *L. rhamnosus.* They have very similar

physiological properties, nutritional requirements and grow under similar environmental conditions. While this group of lactobacilli can be readily distinguished from other members of the *Lactobacillus* genus by fermentation profiles (Hammes *et al.,* 1991), it is not possible to unequivocally distinguish between these three species on this basis.

1.3.2. Taxonomic debate of **Lactobacillus casei** *group*

The taxonomic position of the *L. casei* group has been the subject of some debate. Bergey's Manual of Systematic Bacteriology lists four subspecies of *L. casei* (Kandler and Weiss, 1986) and was reclassified in three species by Collins *et al*. (1989) on the basis of DNA homology data. Mori *et al.* (1997) reported sequences from the 16S rRNA that allows differentiation of these species. So protocols for bacterial typing using polymerase chain reaction (PCR) techniques are becoming increasingly valuable.

Though increasing use of gene sequences for taxonomy, classification of *Lactobacillus* isolates has begun to emerge in a more stable fashion. This has been supported, where appropriate, by biochemical, morphological, and physiological traits. Review of the literature concerning *Lactobacillus casei, Lactobacillus paracasei, Lactobacillus rhamnosus* and *Lactobacillus zeae* reveals that the classification of these bacteria has been controversial and that only recently proposals have been made to handle the ongoing controversy. Given the importance of these bacteria as probiotics or as fermentation contaminants, it is important to have a universally accepted taxonomy to avoid confusion when identifying, examining, and discussing these bacteria. The essence of the debate over the classification of these *Lactobacillus* species is as follows.

Based on 16S rRNA gene sequences (Collins *et al*, 1989), many subspecies formerly known as *L. casei* were reassigned to other species. *L. casei* subsp. *alactosus* and *L. casei* subsp. *pseudoplantarum* were reassigned to *L. paracasei* subsp. *paracasei*, and *L. casei* subsp. *tolerans*

was renamed *L. paracasei* subsp. *tolerans*. Finally, *L. casei* subsp. *rhamnosus* was elevated to the species level and named *L. rhamnosus*. Soon after, Dellaglio *et al.* (1991) requested that the type designation of *L. casei* ATCC 393 be rejected, the neotype *L. casei* ATCC 334^T be recognized, and the name *L. paracasei* be rejected. Later, Dicks *et al.* (1996) called for become the type strain of the *L. casei* ATCC 393 to be designated *L. zeae.* It also was suggested that *L. casei* ATCC 334^T become the type strain of the *L. casei* spp. That includes all *L. paracasei* isolates (Dicks *et al.,* 1996). Further support for this claim came later from Felis *et al.* (2001) and Dellaglio *et al.,* (2002).

Various studies have supported the need for taxonomic changes in this branch of the genus *Lactobacillus* (Collins *et al.,* 1989, / 1991; Dellaglio *et al.,* 1991; Dicks *et al.,* 1996; Mori *et al.,* 1997; Tynkkynen *et al.,* 1999; Chen *et al.,* 2000). Collectively, these studies indicated that *L. zeae* (basonym *L. casei*) ATCC 393, *L. zeae* (basonym *L. rhamnosus*) ATCC 15820, and *L. zeae* isolates from a common group. The remaining *L. casei* isolates, including ATCC 334^T, and *L. paracasei* isolates from another group, and *L. rhamnosus* isolates, except ATCC 15820, belong to a third group. Although the International Committee on Systematic Bacteriology recently endorsed these findings (Biavati 2001; Klein 2001), the proposed changes are not widely known and therefore have not found common usage.

1.4. Gut Microflora

The human colon is the body's most metabolically active organ. This is because of the resident microbiota, which comprises 10^{12} bacterial cells for every gram of gut contents. There are numerous publications purporting that probiotic are active in the gut after ingestion, others have questioned such claims and the beneficial effects that probiotics are said to confer their hosts.

In terms of the microbiology of different digestive tract areas, there is variability both in terms of composition and activity. The lumen of the human stomach is essentially sterile due to a low gastric pH. However, micro-organisms are known to reside in the mucosal layer that overlies the gastric epithelium. This includes *Helicobacter pylori*, which has attracted a great deal of research interest. This organism uses its flagellae to invade the gastric mucus layer and thereafter adhere to epithelial cells. In conjunction with a production of ammonia, this allows effective colonization of the stomach (Rathbone and Heatley, 1992).

In the small intestine, the transit time of gut contents tends to maintain bacterial numbers at below 10^6 / ml of contents. Intestinal secretions like pancreatic enzymes and physiochemical variables such as pH and E_h also contribute towards the type of microflora that develop. Facultatively anaerobic and aerotolerant bacteria such as streptococci, staphylococci and lactobacilli dominate the upper small gut with bacterial numbers showing a progressive increase.

In comparison to other regions of the gastrointestinal tract, the human large intestine is an extremely complex microbial ecosystem, with at least several hundred different bacterial species being present. The environment is favourable for bacterial growth with a slow transit time, ready availability of nutrients and favourable pH. The vast majority (>90%) of the total cells in the body are present as bacteria in the colon. It is thought that over 60% of the faecal mass exists as

prokaryotic cells. Generally, the various components of the large intestinal microbiota may be considered as exerting pathogenic effects or they may have potential health promoting values. Given that the microbiota has components that are positive for human health, there is currently much interest in the use of diet to specifically increase groups perceived as health promoting. As such, the gastrointestinal flora and its activities are a major focus for functional food developments.

1.4.1 Microbiological aspects of large intestine

The large intestine harbours the largest and most complex microbial ecosystem associated with the human body, consisting of several hundred different strains of anaerobic bacteria, with numbers exceeding 10^{11}/g of intestinal contents (Fooks *et al.*, 1999; McBain and Macfarlane, 1998). This is because of the resident microbiota, which comprises 10^{12} bacterial cells for every gram of gut contents. As the large intestine usually contains about 200 g of contents, there is enormous biological activity. The fact that these activities can be modulated or perhaps even controlled through diet is of high relevance (Gibson *et al*., 2000). The microbiota is involved in the catabolism of a vast range of dietary and endogenously secreted compounds. The products of these biotransformations are often toxicological significance to the host. For example, the occurrence of colon cancer is greatly influenced by diet, while metabolism of dietary components by intestinal bacteria has been demonstrated to be an important factor in tumour initiation. The colonic microflora may be involved in the aetiology of large bowel cancer by chemical modification or activation of a wide variety of chemical agents with carcinogenic or co-carcinogenic potential. Exposure of the intestinal microbiota to potential toxicants may occur due to their presence in the diet by biliary excretion of endogenously metabolised substances into the intestine, enzymic activation of procarcinogens by the gut microflora or by direct production of

mutagenic substances by intestinal microorganisms (McBain and Macfarlane, 1998). In terms of functionality, the human colon is the body's most metabolically active organ.

Besides vertical transmission of microorganisms, the body surfaces mentioned are contaminated during and directly after birth with a variety of microbial strains from the immediate environment (horizontal transmission). A number of these microorganisms will colonise these body surfaces permanently or temporarily, while others disappear. The temporarily colonising strains act as pioneers, which initiate the successive domiciliation of other microorganisms. During time, under normal conditions, the microflora will mature to a balanced composition of many different microorganisms.

The occupation of special niches depends on the local environmental circumstances in these specific habitats. These local conditions are determined by multifactorial interactive processes between the host and the microorganisms. Therefore, the mature composition of the natural microflora is specific to the animal species and even specific to an individual. In general, the natural microflora is called the indigenous microflora of a given species (including autochthonous and allochthonous microorganisms), and the indigenous microflora of a given individual (mainly autochthonous microorganisms), respectively. However, the terminology around symbiosis of microorganisms on body surfaces is often confusing.

Species specificity of the microflora has been shown in several studies. Characterisation of *Lactobacillus* and *Bifidobacterium* from humans and different animal species shows various biotypes. The exchange of *Lactobacillus* from one species to another shows that these bacteria do not colonise mutually and that normalisation of the intestinal microflora do not occur by inoculating germ-free animals with a complex microflora from other animal species. Colonisation in specific habitats within the intestinal tract is demonstrated by the fact that some strains are found in crypts while others are found on epithelial surfaces of the villi and that some

microorganisms (filamentous bacteria) are found on specific sites (columnar epithelium) in the small intestine.

During one's lifetime further adaptation of the indigenous microflora occurs, due to the changing local circumstances on the condition that these changes take place gradually, such as due to ageing, and not abruptly (e.g. by a sudden change in food composition or drug use).

A schematic review of the predominant microflora of human body surfaces has been published by Tannock (1988). In mature microbial ecosystems, most microorganisms are obligately anaerobic, even on surfaces which are in direct contact with the air. The density of the microbial population varies from site to site, but can reach 10^8 bacteria per ml of saliva and 10^{10} per gram contents of the large intestine. The numbers of eukaryotic cells are very low in comparison to prokaryotic cells. The diversity of microbial species or strains is thrilling. Progress in isolation and identification techniques, such as the use of anaerobic chambers, cell-wall analysis and DNA-DNA hybridisation, offer an increasing possibility to discriminate between the different microbial strains, for example, on the gene level.

1.4.1.1. Importance of an indigenous microflora

A well-established and matured indigenous microflora on external and internal body surfaces of animals and man is very stable. The penetration and colonisation of non-indigenous microorganisms from the environment and/or from other animal species (xenochthonous microorganisms) onto these body surfaces is hindered.

The importance of an indigenous microflora in the gut as a natural resistance factor against potential pathogenic microorganisms was already recognised in the 19th century by Metchnikoff during his research on cholera. Many decades later the role of the indigenous microflora received renewed interest after findings in laboratory animals orally treated with antibiotics. The antibiotics caused intestinal disturbances owing to infectious agents. It was

suggested that this effect was induced by suppressing the normal gut microflora. Later on, the protective effect of the normal intestinal microflora in chickens against *Salmonella infantis* infection was shown by Nurmi and Rantala (1973). Colonisation resistance of the gut microflora was further confirmed for *Salmonella* and for other pathogenic bacteria such as *Escherichia coli*, *Clostridium* and *Yersinia enterocolitica*. Although Impey *et al.* (1982) demonstrated a protective effect of 48 selected bacterial strains, the colonisation resistance is most effective when a complete species-species microflora has settled down. This has been demonstrated in `normalisation' studies with germ-free animals inoculated with several dilutions of the total intestinal microflora from normal animals of the same species.

The colonisation resistance induced by an indigenous microflora is partly based on occupation of available niches (competitive inhibition of binding sites) and autogenic regulation factors (e.g. synthesis of fatty acids, hydrogen peroxide, bacteriocins). Another important factor might be the non-specific activation of the immune system. The gut, the mucosa, as well as the skin, have humoral and cellular immune systems which can influence the composition of the microflora (gut microflora). It has been shown that the activity of the immune system of germ-free animals is very low since less γ-globulin, smaller lymph nodes and fewer lymphocytes and phagocytes were found. Activation of macrophages was noticed following the introduction of indigenous microorganisms. In addition to this stimulating effect on non-specific resistance factors, the gut microflora has also an important complimentary function in the digestion of dietary components, such as plant polymers and the synthesis of vitamins (Wood, 1996).

1.4.1.2. Establishment of bifidobacteria in infants

The composition of human fecal flora changes with advancing age (Naidu *et al*., 1999). Initially, the foetus exists in a sterile environment until birth (Mackie *et al*., 1999). After birth it

rapidly becomes colonised by bacteria, especially bifidobacteria (Wolin *et al*., 1998) from the maternal vagina and other environmental sources (Mutai and Tanaka, 1987). In both breast-fed and bottle-fed infants, the large intestine is first colonised by Enterobacteria, Streptococci, including Enterococci, Clostridia on the 1^{st} to 2^{nd} day of life. On the 3^{rd} day, bacteroides, bifidobacteria and clostridia have been isolated from over 40% of infants. Between days 4 and 7, bifidobacteria becomes more predominant accounting for 10^{10}-10^{11} organisms per gram of faeces of breast-fed infants, exceeding enterobacteria by 100-1000 times. Clostridia, bacteroides, streptococci and staphylococci decrease, whereas enterobacteria are the predominant organisms in the bottle-fed infants, exceeding bifidobacteria by about 10 times. Thus, nearly 100% of all bacteria cultured from the stools of breast-fed infants were bifidobacteria. At 1 month of age, bifidobacteria were the most prevalent organisms in both groups but the number of these organisms in the stool of bottle-fed infants was approximately one-tenth that of breast-fed infants. Benno and Mitsuoka (1986) also identified all isolates from the stools of healthy infants during the first week of life down to the species level. A total of 37 different species or biovars were obtained. No *Bifidobacterium* or *Eubacterium* sp. was recovered from any of the neonates on the first and second days of life. *Clostridium paraputrificum* and *Bacteroides fragilis* group were detected from the faeces on the first day of life. From the third to fourth day of age, however, *Bifidobacterium* sp. were recovered from the stools of healthy neonates. The incidence of the *Bacteroides fragilis* group, *Clostridium tertium*, *C. paraputrificum* and *Klebsiella pneumoniae* also increased. *Escherichia coli* was the most common facultative species isolated from the stools of all healthy neonates. The next most common species, *Enterococcus faecalis*, *Streptococcus* and *Staphylococcus epidermis*, appeared on the first day after birth. At the end of the study period, *E. coli*, *E. faecalis*, *S. epidermis* and *Streptococcus* sp. had frequently been isolated from 90% of neonates.

1.4.1.3. Differences of the faecal flora between breast-fed and bottle-fed infants

Tissier's turn of the century microscope observations of the faeces of breast milk-fed infants are still valid, but the situation regarding formula-fed babies appears to have altered with improvements to formula feeds which now resemble, but still do not exactly match, the composition of human milk. Well documented modern studies show that bifidobacteria are just as common and likely to be numerically dominant in the faeces of formula-fed as in breast-fed infants. There is considerable infant-to-infant variation in the population size of particular bacterial genera during the first week of life in both infant groups which may have contributed to the somewhat variable interpretations of the status of the infant microflora reported in the literature. More consistent values are obtained in babies older than one week, however, and realistic comparisons between infant groups are possible.

Colonization of the gastrointestinal tract of newborn infants occurs within a few days of birth (Simon and Gorbach, 1984). The inoculum may be derived either from the mother's vaginal or faecal flora (in a conventional birth) or from the environment (in a caesarian delivery). Initially, facultative bacterial species such as *Escherichia coli* or streptococci, are transferred. These are relatively nutritionally undemanding bacteria. Subsequently, their activities create a highly reduced environment which allows the development of the strictly anaerobic bacteria that will later dominate the colon. Dependent on the type of feeding regime given in early life, there appears to be variability in microflora development. The breast-fed infant has a preponderance of bifidobacteria, which easily outcompete other genera. In contrast, the formula-fed infant has a more complex flora which resembles the adult gut in that bacteroides, clostridia, bifidobacteria, lactobacilli, Gram positive cocci, coliforms and other groups are all represented in fairly equal proportions. The type of delivery, dietary constituents and gestational age influence the colonization pattern. The initial period of bacterial colonization in the colon takes place over

approximately a two-week period. During this period the bacterial colonization is similar for formula- and breast-fed infants. *Escherichia coli* and *Streptococcus* are always the first organism to be detected, at concentrations between 10^8 and 10^{10} organisms per gram of faeces.

Several anaerobic organisms, namely *Bifidobacterium*, *Clostridium* and *Bacteroides*, often then take up residence in the gastrointestinal tract. In breast-fed infants a major decrease in the bacterial populations of *E. coli* and *Streptococcus* then occurs, as well as partial or complete disappearance of *Clostridium* and *Bacteroides* resulting in predominance of *Bifidobacterium*. In formula-fed infants, these reductions or disappearances do not take place, resulting in a more complex flora. The relatively simple flora of the breast-fed baby remains until dietary supplementation occurs. Upon introduction of other foods to the diet of the breast-fed infant there is a return of *E. coli*, *Streptococcus* and *Clostridium* to the faeces. The differences between breast-fed and formula-fed infants disappear. There is then a transition period, which continues into the second year of life in which the intestinal flora evolves to resemble that of the adult.

The classical studies concerning the acquisition of the infant intestinal microflora are generally considered to be those of Tissier (190). He divided the colonization of the intestinal tract of suckled infants into three phases. In the first phase, which consisted of the first few hours of life, the faeces were devoid of microbes. The second phase began between the 10^{th} and 20^{th} hour of life with the detection of a heterogenous collection of microbial types in the faeces. After three days, by which time milk had passed through the length of the intestinal tract, the third colonization phase began. A Gram-positive *Bacillus* became the numerically dominant microbe in the faeces at this stage judged by microscope examination of faecal smears. The other microbial types disappeared in a fairly consistent manner, and from the start of the third and fourth day of life until weaning the collection of microbes in the faeces remained the same.

The faecal flora of breast-fed infants is relatively simple. Bifidobacteria are the dominant organisms, accounting for about 99% (range 85% to more than 99%) of cultivable flora. Coliforms, enterococci and lactobacilli comprise about 1% (range 1-15%) of the faecal flora, while bacteroides, clostridia, and other organisms may be absent or insignificant. The stools have an acid pH (4·5-5·5). Since the work of Tissier, it has been believed that bifidobacteria are found exclusively in faeces of breast-fed infants, whereas in bottle-fed infants *Lactobacillus acidophilus* is the most commonly found organism. Twenty-one genera and 103 species or biovars of microorganisms were isolated from the faeces of the breast-fed infants and 20 genera and 97 species or biovars from the bottle-fed infants. The organism that showed the highest number and the highest frequency of occurrence in both groups was *Bifidobacteium breve. Bifidobacterium infantis*, which was formerly the most prevalent *Bifidobacterium* species in baby faeces, was never isolated at this time.

1.4.1.4. The faecal flora of children and adults

The compositions of the bacterial flora in the large intestine and faeces of different age groups may differ. The most prevalent bacteria in the faeces of infants and adults are obligate anaerobes, while facultative anaerobes are generally expected to account for less than 10^3 of anaerobe numbers. During weaning, bifidobacteria decrease by 1 log, the species and biovars alter from infant-type to adult-type, and a remarkable proliferation of bacteroides, eubacteria, peptostreptococcaceae, and clostridia occur. The faecal flora of children closely resembles that of adults, where the numbers of bacteroidaceae, eubacteria, peptococcaceae, and usually clostridia outnumbered bifidobacteria, which constitute 5-10% of the total flora. The counts of Enterobacteriaceae and Streptococci decrease to less than 10^8 per gram of faeces, but the counts are usually less than 10^7 per gram of faeces (Mitsuoka & Hayakawa, 1973).

In adults, the flora of the large intestine is more complex that that of children. The stools of adults have a low redox potential (E_h), a neutral or slightly alkaline pH (6·0-7·0 or above), a typical odour and colour, and they contain relatively large amounts of putrefactive products, such as ammonia, amines, phenols, and degraded bile acids. A comparison of faecal flora in healthy adults and elderly persons is shown in Table 1.6. below.

Table 1.6. Comparison of faecal flora of healthy adults and elderly persons (Wood, 1996)

Intestinal Flora	**Healthy adults (42)[a]**		**Aged (54)[b]**	
Total counts	11.2 ± 0.2[b]		11.1 ± 0.2	
Bacteroidaceae	10.9 ± 0.2	(100)[c]	10.9 ± 0.3	(100)[c]
Eubacterium	10.4 ± 0.4	(100)	10.1 ± 0.7	(100)
Peptococcaceae	10.2 ± 0.3	(100)	10.0 ± 0.7	(100)
Bifidobacterium	10.0 ± 0.8	(100)	9.4 ± 0.8	(85.2)
Veillonella	7.4 ± 1.2	(78.6)	5.2 ± 2.0	(61.1)
Megasphaera	9.0 ± 0.5	(33.3)	8.5 ± 1.1	(16.7)
Curved rods	9.7 ± 0.5	(23.8)	9.3 ± 0.9	(29.6)
Clostridium perfringens	4.4 ± 1.2	(45.5)	6.6 ± 2.0	(83.3)
Clostridium – other	9.5 ± 0.5	(66.7)	9.6 ± 0.8	(100)
Lactobacillus	5.8 ± 2.1	(90.5)	7.5 ± 1.7	(98.1)
Enterobacteriaceae	7.8 ± 0.8	(100)	8.2 ± 1.3	(100)
Streptococcus	7.9 ± 1.4	(100)	7.4 ± 1.6	(100)
Staphylococcus	3.1 ± 0.7	(78.6)	3.8 ± 1.2	(44.4)
Corynebacterium	5.3 ± 2.2	(35.7)	4.7 ± 2.0	(7.4)
Yeasts	3.9 ± 1.6	(42.9)	4.7 ± 1.5	(72.2)

[a] Number of subjects examined.

[b] Mean ± SD of the bacterial counts $\log_{10}$ per gram wet faeces.

[c] Frequency of occurrence (%)

1.4.1.5. Faecal flora of elderly persons

In adults, little is known about the influence of the ageing process on faecal microflora (Andrieux *et al*., 2002). Research conducted by Mitsouka & Hayakawa (1973) compared the faecal flora of healthy adults and elderly persons. In elderly persons, bifidobacteria decrease or diminish, clostridia including *C. perfringens* significantly increase, and lactobacilli, streptococci and Enterobacteriaceae also increase (Hopkins and Macfarlane, 2002).

1.4.1.6. Activity of bacteria in the gastrointestinal tract

In terms of the microbiology of different digestive tract areas, there is variability both in terms of composition and activity. The lumen of the human stomach is essentially sterile due to a low gastric pH. However, micro-organisms are known to reside in the mucosal layer that overlies the gastric epithelium. In the small intestine, the transit time of gut contents tends to maintain bacterial numbers at below 10^6/ml contents. Intestinal secretions like pancreatic enzymes and physiochemical variables such as pH and E_h also contribute towards the type of microflora that develops. The upper small gut is dominated by facultatively anaerobic and aero-tolerant bacteria such as streptococci, staphylococci and lactobacilli, with bacterial numbers showing a progressive increase.

1.4.1.7. Gastrointestinal bacteria influenced by diet

Most work on the microbes of the large bowel of adults has concentrated on the analysis of fecal samples. The composition of the fecal microbiota has been the subject of many investigations and has been summarized in numerous reviews. Most studies focus on enumeration of the major groups of microbes with some studies characterised to the genus level. Few workers have been as thorough as Moore and Holdeman (1974) who identified the species level wherever possible.

Populations of faecal bacteria constitute a major proportion (approximately 50%) of feces (Tomomatsu, 1994). Findings by Moore and Holdeman (1974) noted that despite considerably variation in diet and health, numbers of the major bacterial groups in all subjects were remarkably similar. Bacteroides were found in highest numbers in all fecal numbers. The most frequently isolated anaerobic cocci were peptostreptococci, ruminococci, viellonella, and anaerobic streptococci, with Peptostreptococcus being the most commonly isolated species. Of the anaerobic Gram-positive nonsporing rods, eubacteria were found in highest numbers.

1.4.2. Structure and function of the gastrointestinal tract (GI)

The gastrointestinal tract is a tube extending from the lips to the anus and is divided into various well-defined anatomical regions. The digestive and absorptive functions are well known but, in addition to being an organ in the body, the intestine acts as a container for the most intimate portion of the chemical environment. Assimilation of food is not the only physiological function of the alimentary tract. It is also concerned with the excretion of chemical waste, the control of body metabolism and immune response. Furthermore, the gut harbours a complex ecosystem.

1.4.3. Bacterial fermentations in the large intestine

It is clear that a complex, resident gut flora is present in humans. While the transit of residual foodstuffs through the stomach and small intestine is probably too rapid for the microflora to exert a significant impact, this slows markedly in the colon. The average transit time is around 70 h, but can be higher. As such, colonic micro-organisms have ample opportunity to degrade available substrates. These may be derived from either the diet or by endogenous secretions. Fermentations by gut bacteria consist of a series of energy yielding reactions that do

not use oxygen in the respiratory chains. The electron acceptors may be organic (e.g. some products of the fermentations) or inorganic (e.g. sulphate, nitrate). As carbohydrates form the principal precursors for fermentation, ATP is usually formed through substrate level phosphorylation by saccharolytic micro-organisms. The fermentation process in the large gut is influenced by a variety of physical, chemical, biological and environmental factors. Factors affecting fermentation in the large intestine are presented in Table 1.7.

Table 1.7. Factors affecting fermentation in the human large intestine (Gibson and Fuller, 2000)

- Amount of substrate available for fermentation
- Colonic transit time
- Physical form of the substrate
- Chemical composition of the substrate
- pH of gut contents
- Composition of the gut microbiota with respect to species diversity and relative numbers of different types of bacteria
- Ecological factors including competitive and cooperative interactions between bacteria
- Rates of depolymerisation of substrates
- Substrate specificities and catabolite regulatory mechanisms of individual gut species
- Fermentation strategies of individual substrate utilising bacteria
- Antibiotic therapy
- Availability of inorganic electron acceptors

Major substrates available for the colonic fermentation are starches that, for various reasons, are resistant to the actions of pancreatic amylases and can be degraded by bacterial enzymes as well as dietary fibers like pectins and xylans. Other carbohydrate sources available for fermentation in lower concentrations include oligosaccharides and a variety of sugars and non-absorbable sugar alcohols.

1.4.3.1. Metabolites produced in the large intestine

In terms of end products, a variety of different metabolites arise. Predominant among these are the short chain fatty acids (SCFA), acetate, propionate and butyrate. The majority are

absorbed into the bloodstream and can be further metabolised systemically. Transport to, and further metabolism of SCFA in the liver, muscle or other peripheral tissues is thought to contribute about 7 to 8% of host daily energy requirements. Other products include metabolites, such as ethanol, pyruvate and lactate, which are mostly further converted to SCFA and therefore not allowed to accumulate to any significant level in the large bowel. The resulting end products due to carbohydrate fermentation with the major bacterial groups involved and metabolic fate are presented in Table 1.8.

Table 1.8. Predominant products of carbohydrate metabolism in the human colon (Gibson and Fuller, 2000)

End product	**Bacterial group involved**	**Metabolic fate**
Acetate	*Bacteroides, bifidobacteria, eubacteria, lactobacilli, clostridia, ruminococci, peptococci, Veillonella, peptostreptococci, propionibacteria, fusobacteria, butyrivibrio*	Metabolised in muscle, kidney, heart and brain
Propionate	*Bacteroides, propionibacteria, veillonella*	Cleared by the liver; possible gluceogenic precursor; suppresses cholesterol synthesis
Butyrate	*Clostridia, fusobacteria, butyrivibrio, eubacteria, peptostreptococci*	Metabolised by the colonic epithelium; regulator of cell growth and differentiation
Ethanol, succinate, lactate, pyruvate	*Bacteroides, bifidobacteria, Lactobacilli, eubacteria, peptostreptococci, clostridia, ruminococci, actinomycetes, enterococci, fusobacteria*	Absorbed; electron sink products further fermented to SCFA
Hydrogen	*Clostridia, ruminococci, fusobacteria*	Partially excreted in breath; metabolised by hydrogenotrophic bacteria

1.4.4. Cancer of the large intestine

The large intestine is the second most common site for carcinoma in man and faeces from individuals living in Western societies frequently contain mutagenic substances as indicated by the Ames test. There is no general agreement regarding the aetiology of bowel cancer, although factors such as diet, environment and genetics have been implicated. It has been speculated that tumours occur 100 times more often in the hindgut than in the small intestine, indicating that the colonic microbiota plays an important role in carcinogenesis. It has been suggested that a mechanism whereby intestinal bacteria may be involved in these processes is by the production of carcinogenic metabolites from non-toxic precursor molecules, and a variety of hydrolytic and reductive enzymes responsible for carcinogen production are produced by colonic micro-organisms (McBain and Macfarlane, 1998).

1.4.4.1. Conditions favourable for cancer

Colorectal cancer is the second largest cause of cancer deaths in western countries (Singh *et al*., 1997). Studies have suggested the involvement of intestinal microflora in the pathology of colon cancer. Epidemiological and experimental studies provide evidence that nutritional factors play a role in the aetiology of colon cancer (Kulkarni and Reddy, 1994). Lactulose, a disaccharide and keto hydrolytic product of β-galactosidase, is not absorbed in the small intestine. These sugars are used as a substrate by bifidobacteria resulting in an increase in the number of bifidobacteria in faeces (Salminen *et al*., 1993; Gibson *et al*., 1994). These sugars enhance the selective proliferation and colonisation of bifidobacteria. Shifts in the colonic flora, i.e. an increase in anaerobes and a decrease in aerobes have been found in populations which are at increased risk of colon cancer. With respect to colon carcinogenesis, the fermentation of carbohydrate and dietary fibre by colonic bacteria to short chain fatty acids is of major interest.

An acidic pH level in the colonic lumen caused by increased production of short chain fatty acids inhibits the bacterial degradation of primary to secondary bile acids, which have been shown to promote colon cancer in carcinogen-treated rats.

1.4.4.2. Bacterial prevention of cancer

Many reported studies have shown the beneficial effect of consuming specific lactic acid bacteria in the prevention of chronic conditions such as cardiovascular disease and cancer. These lactic cultures which are primarily used for fermentation of milk and other dairy products have shown to possess antimutagenic and anticarcinogenic properties and from epidemiological and experimental studies reduce certain types of cancer and inhibit tumour growth (Singh *et al*., 1997). Japanese research by Kubota (1990) found that colon cancer incidence was lowest when the colonic population of bifidobacteria was highest and that of *Clostridium perfringens* was lowest.

Results of the study carried out by Challa *et al*. (1997) indicate that *Bifidobacterium* and lactulose exert an additive antitumorigenic effect in rat colon. While the mechanism of inhibition of colon carcinogenesis by dietary *B. longum* has not been clarified, it is likely that the effect of lactic bacteria can proceed through diverse mechanisms. These may include the alteration of physiological conditions in the colon affecting the metabolic activity of intestinal microflora, the action of bile acids, and to quantitative and/or qualitative alterations in the bile acid-degrading bacteria. The species of lactobacilli and *Bifidobacterium*, most often suggested as beneficial dietary supplements, have all been reported to exert anatagonistic actions toward several enteropathogenic organisms in the intestine such as *Escherichia coli* and *Clostridium perfringens. C. perfringens* and other enteropathogenic anaerobic bacteria contain high levels of 7α-dehydroxylase, which is an important enzyme in the formation of the secondary bile acids from

the primary bile acids in the colon. These secondary bile acids have been shown to play a role as tumour promoters in the colon. Evidence of correlations between the incidence of colon cancer and the number of bacteria per gram of faeces possessing 7α-dehydroxylase enzyme activity have been found in humans. It is then possible to assume that dietary lactic cultures modulate the metabolic activity of intestinal microflora and the activity of 7α-dehydroxylase thereby producing lower levels of secondary bile acids in the colon (Reddy *et al*., 1993).

1.4.5. Natural microflora in gastrointestinal tract

Many different types of bacteria representing most bacterial groups have at some time been isolated from the intestine. Those isolated most frequently can be considered as members of the resident flora or as contaminants from the environment. The number of bacterial groups that may be detected is related to the methods used for their detection. Very few investigators have attempted a systematic investigation of the intestinal bacteria and so any list of the species present in the gut must be provisional.

Numerically, the most important genus of intestinal bacteria in animals and man is *Bacteroides*. This along with *Fusobacterium*, which contains pathogenic species, and *Leptotrichia*, which is also found in the mouth, comprise the family *Bacteroidaceae* which also contains members of the former genus *Sphaerophorus*. These are all Gram-negative, strictly anaerobic, non-sporing rods, although some may show varying degrees of polymorphism. *B. fragilis* is ubiquitous in animals and man.

Amongst the Gram-positive, non-sporing rods several genera are numerically important in the gut. Obligately anaerobic types include *Propionibacterium* (mainly *P. acnes*), *Eubacterium* and *Bifidobacterium*, including *B. bifidum* and *B. infantis* from the faeces of breast-fed infants. Among the facultative anaerobes the genus *Lactobacillus* contains many species occurring in the

gut of most warm-blooded animals. Although numerically important throughout the alimentary tract their ecological significance has not been conclusively elucidated.

Several types of spore-forming rods and cocci are normal inhabitants of the gut. The genus *Clostridium* is probably the most ubiquitous. Others such as *C. perfringens, C. bifermentans* and *C. tetani* are found regularly, albeit in relatively low numbers but are of significance in humans.

Facultative and obligately anaerobic Gram-positive cocci are numerically important in the gut. The facultatively anaerobic streptococci are well represented by many species from Lancefield group D including *Streptococcus faecalis, S. bovis* and *S. equinus* and come from group K such as *S. salivarius* which is usually associated with the mouth. Gram-negative anaerobic cocci include the closely related genera *Veillonella* which utilises lactic acid and *Acidaminococcus* which can utilise amino acids as a sole energy source.

Although they are not numerically important, the Gram-negative facultatively anaerobic rods include a number of very important pathogens. Members of the related genera *Vibrio* and *Campylobacter* cause enteric disease in man and animals. The taxonomy of the *Enterobacteriaceae* is complex and while some genera such as *Proteus* and *Klebsiella* and many serotypes of *E. coli* and *Salmonella* are commensal in animals particular biotypes of the latter two genera are major animal pathogens, particularly affecting the young. Although *Shigella* causes dysentery in man it may be found existing as a commensal in the gut of other warm-blooded animals.

The significance of the presence of yeasts and moulds in the gut is uncertain. Some yeasts associate with the murine stomach wall but in general they are thought to be transient contaminants. A total bacterial count of major groups of bacteria within each section of the gastrointestinal tract is presented in Table 1.9.

Table 1.9. Distribution of human gastrointestinal flora in different segments of the gastrointestinal tract (Simon and Gorbach, 1984)

	Stomach	Jejunum	Ileum	Faeces
otal bacterial count	$0\text{-}10^{3}$[a]	$0\text{-}10^{5}$	$10^{3}\text{-}10^{7}$	$10^{10}\text{-}10^{12}$
Aerobic or facultative anaerobic bacteria				
Enterobacteria	$0\text{-}10^{2}$	$0\text{-}10^{3}$	$10^{2}\text{-}10^{6}$	$10^{4}\text{-}10^{10}$
Streptococci	$0\text{-}10^{3}$	$0\text{-}10^{4}$	$10^{2}\text{-}10^{6}$	$10^{5}\text{-}10^{10}$
Staphylococci	$0\text{-}10^{2}$	$0\text{-}10^{3}$	$10^{2}\text{-}10^{5}$	$10^{4}\text{-}10^{7}$
Lactobacilli	$0\text{-}10^{3}$	$0\text{-}10^{4}$	$10^{2}\text{-}10^{5}$	$10^{6}\text{-}10^{10}$
Fungi	$0\text{-}10^{2}$	$0\text{-}10^{2}$	$10^{2}\text{-}10^{3}$	$10^{2}\text{-}10^{6}$
Anaerobic bacteria				
Bacteroides	Rare	$0\text{-}10^{2}$	$10^{3}\text{-}10^{7}$	$10^{10}\text{-}10^{12}$
Bifidobacteria	Rare	$0\text{-}10^{3}$	$10^{3}\text{-}10^{5}$	$10^{8}\text{-}10^{12}$
Gram-positive cocci	Rare	$0\text{-}10^{3}$	$10^{2}\text{-}10^{5}$	$10^{8}\text{-}10^{11}$
Clostridia	Rare	Rare	$10^{2}\text{-}10^{4}$	$10^{6}\text{-}10^{11}$
Eubacteria	Rare	Rare	Rare	$10^{9}\text{-}10^{12}$

[a] Number per gram of intestinal contents.

Presented in Table 1.10 is a detailed description of bacterial species isolated from the healthy human intestine grouped by Gram strain, oxygen requirements and morphology.

Table 1.10. Some bacterial species isolated from the healthy human intestine (Drassar and Barrow, 1985).

Anaerobic bacteria Gram-negative anaerobic rods	**Gram-positive anaerobic rods**	**Gram-positive anaerobic cocci**	**Facultatively anaerobic bacteria**
Bacteroides assacharolyticu,	*Clostridium bejerinki*	*Peptococcus*	*Streptococcus faecalis*
Bacteroides capillosus	*Clostridium butyricum*	*asaccharolyticus*	*Streptococcus faecium*
Bacteroides coagulans	*Clostridium cadaveris*	*Peptococcus magnus*	*Streptococcus bovis*
Bacteroides distasonis	*Clostridium celatum*	*Peptococcus prevotii*	*Streptococcus agalactiae*
Bacteroides eggerthii	*Clostridium clostridiiforme*	*Ruminococcus albus*	*Streptococcus anginosus*
Bacteroides fragilis	*Clostridium difficile*	*Ruminococcus bromii*	*Streptococcus avium*
Bacteroides furcosus	*Clostridium inoculum*	*Ruminococcus*	*Streptococcus cremoris*
Bacteroides hypermegas	*Clostridium leptum*	*flavefaciens*	*Streptococcus equisimilius*
Bacteroides acteroides	*Clostridium malenominatum*	*Streptococcus*	*Streptococcus lactis*
Bacteroides multiacidus	*Clostridium nexile*	*constellatus*	*Streptococcus mitior*
Bacteroides ovalis	*Clostridium paraputrificum*	*Streptococcus*	*Streptococcus mutans*
Bacteroides ovatus	*Clostridium perfrigens*	*intermedius*	*Streptococcus salivarius*
Bacteroides praeacutus	*Clostridium ramosum*	*Streptococcus*	*Streptococcus sanguis*
Bacteroides putredinis	*Clostridium tertium*	*morbillorium*	*Streptococcus epidermidis*
Bacteroides ruminocola	*Bifidobacterium adolescentis*	*Megasphera elsdenii*	*Lactobacillus casei*
Bacteroides s.s. brevis	*Bifidobacterium angulatum*		*Lactobacillus fermentum*
Bacteroides ruminocola s.s. ruminicola	*Bifidobacterium bifidum*	*Peptostreptococcus productus*	*Lactobacillus leichmannii*
Bacteroides splanchinicus	*Bifidobacterium breve*		*Lactobacillus minutes*
Bacteroides thetaiotaomicron	*Bifidobacterium catenulatum*	*Sarcina ventriculi*	*Lactobacillus plantarum*
Bacteroides uniformis	*Bifidobacterium cornutum*		*Lactobacillus rogosae*
Bacteroides vulgatus	*Bifidobacterium dentium*	*Gemiger formicilis*	*Lactobacillus ruminis*
	Bifidobacterium infantis		*Lactobacillus salivarius*
	Bifidobacterium longum		*Lactobacillus acidophilus*
	Bifidobacterium pseudolongum		*Lactobacillus brevis*
Fusobacterium mortiferum	*Eubacterium aerofacien*	*Acidaminococcus fermantans*	*Enterobacteriaceae*
Fusobacterium naviforme	*Eubacterium contortum*		*Escherichia coli*
Fusobacterium necrogenes	*Eubacterium cylindroids*	*Veillonella parvula*	*Citrobactor freundii*
Fusobacterium nucleatum	*Eubacterium lentum*		*Klebsiella pneumoniae*
Fusobacterium plauti	*Eubacterium limosum*	*Coprococcus cutactus*	*Enterobacter cloacae*
Fusobacterium prausnitzii	*Eubacterium rectale*	*Coprococcus catus*	*Enterobacter aerogenes*
Fusobacterium russi	*Eubacterium ruminantium*	*Coprococcus comes*	*Proteus mirabilis*
Fusobacterium symbiosum	*Eubacterium tenue*		*Proteus morganii*
Fusobacterium varium	*Eubacterium tortuosum*		
	Eubacterium ventriosum		
Leptotrichia buccalis	*Propionbacterium acnes*		
Butyrivibrio fibriosolvens	*Propionbacterium granulosm*		
Sucinimonas amylolytia	*Propionbacterium jensenii*		
Desulfomonas pigra	*Lachnospira multiparus*		
Vibrio succinogenes			

1.4.6. Bacteria influenced by diet

1.4.6.1. Influence of diet on faecal bifidobacterial flora

It is often reported that the compositions of intestinal flora are influenced by diet. The quality of diet can immensely affect human health preventing and reducing susceptibility to particular diseases (Kolida *et al*., 2000; Gibson, 1999). The physical and physiological characteristics of the gastrointestinal tract and its epithelial layer are greatly affected by the presence of a complex microflora whose density varies according to the section of the intestine colonized. The sensitivity of intestinal microorganisms to gastric acid and oxygen largely determines the sites of colonization. Since the oxidation-reduction potential (E_h) varies according to the microbial population level, the microflora itself controls certain aspects of its own environment. Some facultatively anaerobic groups of bacteria, such as the lactobacilli, streptococci and coliform bacteria, are ubiquitous and are distributed throughout most of the tract. Obligately anaerobic bacteria such as *Bacteroides* and *Bifidobacterium* are confined to parts of the gut where E_h values are very low. Such sites include the colon, caecum and the rumen or rumen-like anatomical modifications of the stomach in those animals possessing a fore-gut microbial fermentation.

Although diet is important in determining the qualitative and quantitative composition of the intestinal microflora, it is difficult to demonstrate experimentally. While the adult flora is characteristic of the host species, that of the neonatal mammal is common to a wide range of species since the milk diet produces a common environment in the gut. Characteristic of the gut flora of neonates are low numbers of potentially pathogenic species such as *E. coli*. These low numbers are maintained by the inhibitory effects of specific antibody (mainly IgA) and several non-specific factors including the iron binding protein, lactoferrin.

Because of the complex intestinal flora, adult animals are normally extremely difficult to infect with enteric pathogens. Disturbance or removal of the flora (for example by antibiotics) thus increases susceptibility to colonisation by these organisms. An additional consequence of oral antibiotic administration is that commensal and pathogenic bacteria may become resistant to these drugs by mutation or by transferable drug resistance. Both these problems are of considerable significance to animal and public health. Because of this renewed attempts are being made to induce changes in the intestinal flora of animals and man, beneficial to host health, by feeding normal constituents of the gut flora or fermented milk products.

The faecal flora of nine rural healthy Japanese and eight urban healthy Canadians were compared (Benno *et al*., 1986). The two populations are typical Japanese and Western diets, respectively. The numbers of eubacteria, bifidobacteria, lactobacilli and veillonellae and the frequency of occurrence of bifidobacteria were higher in the Japanese than in the Canadians. Higher numbers of bacteroides and *C. perfringens* were found in the Canadians. Presented in Table 1.11. is the faecal flora of vegetarian and non-vegetarian Seventh Day Adventists comparing the faecal flora of volunteers consuming high- and low-beef diets.

Table 1.11. Faecal microbiota in various dietary groups including seventh-day Adventists who were strictly vegetarian, Japanese who consumed an oriental diet that included fish but no beef, and healthy subjects who consumed a Western diet with relatively large quantities of beef (Gibson and Macfarlane, 1995).

	Strict vegetarian (13)		**Japanese (15)[c]**		**Western (62)**		**Total[d] (141)**	
Microorganisms	%[a]	Mean[b]	%	Mean	%	Mean	%	Mean
Bacterioides	100	11.7	93	10.8	100	11.3	99	11.3
Fusobacterium	0	-	40	8.1	24	8.6	18	8.4
Anaerobic *Streptococci*	8	11.4	60	9.5	32	10.5	34	10.3
Peptococcus	8	11.2	47	9.4	37	10.1	33	10.0
Peptostreptococcus	23	11.1	80	10.2	35	10.2	45	10.1
Ruminococcus	54	10.2	60	10.3	45	10.0	45	10.2
Anaerobic cocci	85	10.3	100	10.7	98	10.6	94	10.7
Actinomyces	31	10.5	0	-	2	5.7	7.8	9.2
Arachnia-propionibacterium	38	10.0	0	-	2	5.5	9.2	8.9
Bifidobacterium	69	10.9	80	9.7	79	10.4	74	10.2
Eubacterium	92	11.0	93	10.6	95	10.6	94	10.7
Lactobacillus	85	11.1	73	9.0	73	9.3	78	9.6
Clostridium	92	9.4	100	9.7	100	10.2	100	9.8
Streptococcus	100	8.6	100	8.7	100	9.1	99	8.9
Gram-negative facultatives	100	8.2	100	9.2	98	8.9	98	8.7
Candida albicans	15	4.9	47	5.6	14	5.4	14.2	5.4
Other yeasts	23	5.6	53	5.8	31	5.2	36.2	5.6
Filamentous fungi	0	-	0	-	3	3.8	3.5	5.9
Bacillus sp.	69	4.2	80	6.2	82	5.0	82.3	5.2
Total[e]	100	12.6	100	11.8	100	12.2	100	12.2

[a]% Positive. [b]Mean count expressed as organisms $\log_{10}$/g dry weight faeces. [c]Number of subjects per dietary group. [d]Total for all 141 subjects including polyp. Colonic cancer, and vegetarians who consume some meat. [e]Total of all microbes detected (including other genera and groups not listed above).

Table 1.12. below contains the 25 most prevalent bacteria species that are present in the faeces of human subjects.

Table 1.12: The 25 most prevalent bacterial species in the faeces of human subjects consuming a Western diet (10^{9-10} bacteria per gram wet weight) (Gibson and Macfarlane, 1995)

Bacteroides vulgatus	*Ruminococcus albus*	*Bifidobacterium adolescentis* A
Bacteroides species, other	*Bacteroides distasonis*	*Bifidobacterium adolescentis* C
Bacteroides fragilis	*Peptostreptococcus intermedius*	*Bacteroides clostridiiformis* ssp. *clostridiiformis*
Bacteroides thetaiotaomicron	*Peptostreptococcus*	*Peptostreptococcus prevotii*
Peptostreptococcus micros	*Peptostreptococcus productus*	*Bifidobacterium infantis* ssp. *liberorum*
Bacillus species (all)	*Eubacterium lentum*	*Clostridium indolis*
Bifidobacterium adolescentis D	*Facultative streptococci, other*	*Enterococcus faecium*
Eubacterium aerofaciensr	*Fusobacterium russii*	*Bifidobacterium longum*

In summary, it seems that no general agreement exists in regard to whether or not the bifidobacterial flora of individuals on high-meat diets differ from those of individuals on low-meat diets. However, these results, which were obtained using a comprehensive method for cultivating intestinal flora, indicated that a Japanese-style diet is superior to a western-style diet from the viewpoint of bifidobacteria in the intestinal flora.

The characteristics of particular genera commonly found in human faeces are presented in Table 1.13 including metabolic products and metabolic processes.

Table 1.13: Characteristics of bacterial genera commonly detected in human faeces (Tannock,

Genera	Characteristic
Bacteroides	Gram-negative, nonspore-forming bacilli. Obligate anaerobe. Metabolic products include combinations of acetic, succinic, lactic, formic or propionic acids. If *N*-butyric acid is produced, isobutyric and isovaleric acids are also present.
Bifidobacterium	Gram-positive, nonspore-forming, nonmotile bacilli sometimes club-shaped or spatulated extremities. Obligate anaerobe. Acetic and lactic acids are produced primarily in the molar ratio of 3:2. Glucose is degraded exclusively and characteristically by the fructose-6-phosphate `shunt' metabolic pathway.
Clostridium	Gram-positive bacilli that form endoscopes. Obligate anaerobe.
Enterococcus	Gram-positive cocci. Facultative anaerobe. Lancefield group D. Can grow in 6.5% NaCl broth and in broth at pH 9.6.
Eubacterium	Gram-positive bacilli, nonspore-forming. Obligate anaerobe. Produces mixtures of organic acids including butyric, acetic and formic acids.
Fusobacterium	Gram-negative, nonspore-forming bacilli. Obligate anaerobe. *N*-butyric acid is produced, but isobutyric and isovaleric acids are not.
Peptostreptococcus	Gram-positive cocci. Obligate anaerobe. Can metabolise peptone and amino acids.
Ruminococcus	Gram-positive cocci. Obligate anaerobe. Amino acids and peptides are not fermented. Fermentation of carbohydrate produces acetic, succinic and lactic acids, ethanol, carbon dioxide and hydrogen.

1.5. Probiotics

One manner in which modulation of the gut microbiota composition has been attempted is through the use of live microbial dietary additions, as probiotics. The word probiotic is translated from the Greek meaning ‘for life’. An early definition was given by Parker (1974): ‘Organisms and substances which contribute to intestinal microbial balance.’ However, this was subsequently refined by Fuller (1989) as: ‘a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance.’ This latter version is the most widely used definition and has gained widespread scientific acceptability. A probiotic would therefore incorporate living micro-organisms, seen as beneficial for gut health, into diet.

Probiotics has a long history. In fact, the first records of intake of bacterial drinks by humans are over 2000 years old. However, at the beginning of this century probiotics were first put onto a scientific basis by the work of Metchnikoff at the Pasteur Institute in Paris. Metchnikoff (1907) observed longevity in Bulgarian peasants and associated this with their elevated intake of soured milks. During these studies, he hypothesized that the normal gut Microflora could exert adverse effects on the host and that consumption of certain bacteria could reverse this effect. Metchnikoff refined the treatment by using pure cultures of what is now called *Lactobacillus delbruckeii* subsp. *bulgaricus*, which, with *Streptococcus salivarius* subsp. *thermophilus*, is used to ferment milk in the production of traditional yoghurt.

Subsequent research has been directed towards the use of intestinal isolates of bacteria as probiotics (Fernandes *et al*., 1987). Over the years many species of micro-organisms have been used. They mainly consist of lactic acid producing bacteria (lactobacilli, streptococci, enterococci, lactococci, bifidobacteria) but also *Bacillus* spp. and fungi such as *Saccharomyces* spp. and *Aspergillus* spp.

Despite the very widespread use of probiotics, the approach may have some difficulties. The bacteria used are usually anaerobic and do not relish extremes of temperature. To be effective, probiotic must be amenable to preparation in a viable form at a large scale. During use and under storage the probiotic should remain viable and stable, and be able to survive in the intestinal ecosystem, and be able to survive in the ecosystem, and the host animal should gain beneficially from harbouring the probiotic. It is therefore proposed that the exogenous bacteria reach the intestine in an intact and viable form, and establish therein and exert their advantageous properties. In order to do so, microbes must overcome a number of physical and chemical barriers in the gastrointestinal tract. These include gastric acidity and bile acid secrection. Moreover, on reaching the colon the probiotics may be in some sort of stressed state that would probably compromise chances of survival.

1.5.1. Properties required for probiotics to be effective in nutritional and therapeutic settings

A probiotic can be used exogenously or endogenously to enhance nutritional status and/or the health of the host. In the case of exogenous use, microorganisms are most commonly used to ferment various foods and by this process can preserve and make nutrients bioavailable. In addition, microorganisms can metabolise sugars, such as lactose in yoghurt, making this food more acceptable for consumption by individuals suffering from lactose intolerance. However, the most interesting properties that probiotics acting exogenously can have is the production of substances that may be antibiotics, anticarcinogens or have other pharmaceutical properties. The properties required for exogenously derived benefits from probiotics are the ability to grow in the food or the media in which the organism is placed, and the specific metabolic properties which result in the potential beneficial effects stated above. The selection of organisms that can be helpful therapeutically and nutritionally would be based on specific properties that are desired.

This can be achieved by either classical biological selection techniques or genetic engineering. Probiotics that are ingested by the host and exert their favourable properties by virtue of residing in the gastrointestinal tract have to have certain properties in order to exert an effect.

1.5.1.1. Requirements for probiotics

It is of high importance that the probiotic strain can survive the location where it is presumed to be active. For a longer and perhaps higher activity, it is necessary that the strain can proliferate and colonise at this specific location. Probably only host-specific microbial strains are able to compete with the indigenous microflora and to colonise the niches. Besides, the probiotic strain must be tolerated by the immune system and not provoke the formation of antibodies against the probiotic strain. So, the host must be immuno-tolerant to the probiotic. On the other hand, the probiotic strain can act as an adjuvant and stimulate the immune system against pathogenic microorganisms. It goes without saying that a probiotic has to be harmless to the host: there must be no local or general pathogenic, allergic or mutagenic/carcinogenic reactions provoked by the microorganism itself, its fermentation products or its cell components after decrease of the bacteria.

For the maintenance of its favourable properties the strain must be genetically stable. For the production of probiotics it is important that the microorganisms multiply rapidly and densely on relatively cheap nutrients and that they remain viable during processing and storage. Besides the specific beneficial property, these general requirements must be considered in developing new probiotics, but also for determining the scientific value of a claimed probiotic. A number of these requirements can be screened during *in vitro* experiments. It is advised of the drawing up of a decision-tree for the minimal requirements which can be tested *in vitro*, such as culture conditions and viability of the probiotic strains during processing and storage; sensitivity to low pH values, gastric juice, bile, pancreas, intestinal juice and intestinal or respiratory mucus;

adherence to isolated cells or cell cultures and interactions with other (pathogenic) microorganisms. If these *in vitro* experiments are successful, further research can be performed during *in vivo* experiments in animals or humans. Requirements of probiotics that are important for their use in humans are presented in Table 1.14.

Table 1.14. Requirements of probiotics (Salminen and von Wright, 1998)

- Survival of the environmental conditions on the location where it must be active
- Proliferation and/or colonisation on the location where it is active
- No immune reaction against the probiotic strain
- No pathogenic, toxic, allergic, mutagenic or carcinogenic reaction by the probiotic strain itself, its fermentation products or its cell components after decrease of the bacteria
- Genetically stable, no plasmid transfer
- Easy and reproducible production
- Viable during processing and storage

1.5.2. Claimed beneficial properties of probiotics

So far, it was shown that the indigenous microflora is host-specific and location-specific, very complex in composition and that it has beneficial properties to the host. However, it is not precisely known which species of microorganisms play the principal part in these beneficial properties. For man it is suggested that specific microbial strains could play an important role in;

- formation or reconstruction of a well-balanced indigenous intestinal and/or respiratory microflora, for example, in newborn children during admission to an ICU;
- after gastrointestinal decontamination by antibiotics in connection with bone-marrow transplantation;
- improving the colonisation resistance of the indigenous microflora of the intestinal, respiratory and urogenital tracts;
- lowering the serum cholesterol level;
- inhibiting the mutagenicity of the intestinal contents and reducing the incidence of intestinal tumours;

- non-specific interactions with the immune system;
- metabolising lactose and hence reducing lactose intolerance (Jiang *et al*. 1996);
- improving the absorption of calcium and hence inhibiting decalcification of the bones in elderly people;
- synthesis of vitamins and pre-digestion of proteins.

Probiotic foods are becoming increasingly popular. A number of health benefits have been claimed for *Bifidobacterium* sp. and therefore inclusion of these organisms in the diet is considered to be important in maintaining good health (Champagne *et al*., 1996). Probiotics have anticarcinogenic properties, a specific probiotic effect, which are of three types: (1) elimination of procarcinogens; (2) modulation of procarcinogenic enzymes; and (3) tumour suppression (Wollowski *et al*., 2001; Grill *et al*., 1995a). Furthermore, consumption of these organisms is an ideal method to re-establish the balance in the intestinal flora after antibiotic treatment (Gibson *et al*., 1994). There is a growing agreement relating to the beneficial aspects of specific dairy products such as fermented milk and yoghurt and of bacterial cultures that ferment the dairy products in human and animal nutrition. Experimental and epidemiological studies provide evidence that fermented milk and bacterial cultures that are routinely used to ferment the milk reduce the risk of certain types of cancer and inhibit the growth of certain tumours and tumour cells (Reddy and Rivenson, 1993).

Many health promoting effects have been attributed to certain *Bifidobacterium* sp. (Rolfe, 2000). These include reduction of ammonia levels, stimulation of the immune system, alleviation of lactose intolerance and prevention of gastrointestinal disorders (O'Sullivan, 1996). Several probiotic bacteria have been introduced in the market and the range of products in which probiotic bacteria are added is increasing. However, many of the prophylactic and therapeutic

properties of these foods containing bifidobacteria are a matter of speculation because there are inherent difficulties in obtaining definitive evidence for proposed effects of ingesting bifidobacteria.

1.5.2.1. Viability of probiotic organisms

Microorganisms introduced orally have to, at least, transiently survive in the stomach and small intestine. Although this appears to be a rather minimal requirement, many bacteria including the yoghurt-producing bacteria *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* often do not survive to reach the lower small intestine. The reason for this appears to be low pH of the stomach. In fasting individuals, the pH of the stomach is between 1.0 and 2.0 and most microorganisms, including lactobacilli, can only survive from 30 seconds to several minutes under these conditions. Therefore, in order for a probiotic to be effective, even the selection of strains that can survive in acid at pH 3.0 for sometime would have to be introduced in a buffered system such as milk, yoghurt or other food.

1.5.2.2. Antimicrobial properties

As indicated previously, the intestinal microflora is a complex ecosystem. Introducing new organisms into this highly competitive environment is difficult. Thus organisms that can produce a product or products that will inhibit the growth or kill existing organisms in the intestinal milieu have a distinct advantage. The growth media filtrates and sonicates from the bacterial cells of prospective probiotics should be tested for bactericidal and bacteriostatic activity in well-plates against a wide variety of pathogens. The ability of probiotics to establish in the gastrointestinal tract will be enhanced by their ability to eliminate competitors. Table 1.15 presents some examples of antimicrobial-producing organisms.

Table 1.15. Antimicrobial substances (Fuller, 1992)

Probiotic	**Compound**
Lactobacillus GG	Wide spectrum antibiotic
L. acidophilus	*Acidolin, Acidophilin, Lactocidin*
L. delbrueckii ssp. *bulgaricus*	*Bulgarican*
L. plantarum	*Lactolin*
L. brevis	*Lactobacillin, Lactobrevin*
L. reuteri	*Reuterin*

1.5.2.3. Acid and bile tolerance

One of the most important criteria for selection of probiotic organisms is their ability to survive in the acidic environment of the product and in the stomach, where the pH can reach as low as 1.5. Similarly, the organisms must be able to survive in the bile concentrations encountered in the intestine. Lankaputhra and Shah (1995) showed that, among several strains of *L. acidophilus* and *Bifidobacterium* sp. studied, only a few strains survived under the acidic conditions and bile concentrations normally encountered in fermented products and in the gastrointestinal tract, respectively. Therefore, it cannot be generalised that all probiotic strains are acid and bile tolerant. Clark *et al*. (1993) and Lankaputhra and Shah (1995) showed that *Bifidobacterium longum* survives better in acidic conditions and is able to tolerate a bile concentration as high as 4%. Acid and bile tolerance is strain dependent, and care should be taken to select strains based on these attributes.

1.5.2.4. Antagonism among bacteria

Bifidobacteria produce acetic and lactic acids in a molar ratio of 3:2. *L. acidophilus* and *L. casei* produce lactic acid as the main end product of fermentation. In addition to lactic and acetic acids, probiotic organisms produce other acids, such as hippuric and citric acid. Lactic acid bacteria also produce hydrogen peroxide, diacetyl and bacteriocin as antimicrobial substances. These inhibitory substances create antagonistic environments for foodborne pathogens and

spoilage organisms. Yoghurt bacteria are reported to produce bacteriocin against probiotic bacteria and vice versa (Dave and Shah, 1997).

1.5.2.5. Anticarcinogenic properties

In the last two decades, the number of people suffering from colon cancer has been gradually increasing, particularly in industrialised countries (Moore and Moore, 1995; Hidaka *et al*., 1991). Studies by Goldin and Gorbach (1981, 1984a) have indicated that diet and antibiotics can lower the generation of carcinogens in the colon and reduce chemically induced tumours. These effects appear to be mediated through the intestinal microflora. Additional studies have shown that the introduction of *L. acidophilus* into the diet lowered the incidence of chemically induced colon tumours in rats (Goldin and Gorbach, 1980). A possible mechanism for these anticancer effects relies on inhibiting intestinal bacterial enzymes that convert procarcinogens to more proximal carcinogens. This technique can be expanded in the future by testing probiotics for their ability to inhibit the growth or organisms normally found in the flora that have high activities of enzymes such as β-glucuronidase (Reddy *et al*., 1974), nitroreductase, azoreductase and β-glycosidase or the capability for nitrososation. The ability of probiotics to deactivate faecal mutagens can also be a marker used to introduce organisms that lower cancer risk.

1.5.2.6. Adherence of probiotic bacteria

It is not clear if adhesion to the intestinal epithelium is essential for the persistence of a probiotic in the human intestinal tract. However, adhesion seems to be a property that enhances long-term survival. The ability of microorganisms to adhere to epithelial cells is to a large extent species specific, although this may be relative. Screening of organisms for their ability to survive in the human gastrointestinal tract is not difficult. The selection of human bacterial isolates will enhance the possibility of finding organisms that will survive. The isolates can then be tested by

administering orally between 10^9 and 10^{11} viable organism in a single dose with an appropriate buffering agent and the bacterial counts of the specific organism are then measured in the faeces over a several week period. This technique is most successful if the natural flora does not contain the organism being tested or only in small numbers. The first question of transient survival can be established in 48 to 96 h. The evaluation of the ability of the organism to permanently establish in the gastrointestinal tract, by proliferation, can be established by continuous appearance in the faeces over several weeks to several months. The faecal counts should exceed 10^6/g of faeces. The application of this screen for selecting probiotics should be encouraged in the future. There are several tests for determining if a prospective probiotic can bind to intestinal epithelium. Radiolabelling the microorganisms with an amino acid and then counting for adhering radioactivity in either ileal cells recovered from ileostroma effluent or from buccal cells obtained by gently scraping the inside of the cheek are effective methods. Good adhesion properties should enhance the possibility of long-term survival of the organism in the intestinal tract by countering the peristaltic action of the intestine.

1.5.2.7. Immunological enhancement

In recent years there have been several reports indicating that lactobacilli used in dairy products can enhance the immune response of the host. Organisms that have been identified as having this property are *Bifidobacterium longum*, *L. acidophilus, L. casei* subsp. *rhamnosum* and *L. helveticus* (Isolauri *et al*., 2001b). In the future, prospective probiotics, in the appropriate settings (anticancer or infection resistance), should be tested for enhancement of the immunological response. The measurements that should be considered are lymphocyte proliferation, interleukin 1, 2 and 6, tumour necrosis factor, prostaglandin E production and serum total protein, albumin, globulin and gamma interferon.

1.5.2.8. Cholesterol lowering

Experiments by Gilliland *et al.* (1985) have shown that dietary elevation of plasma cholesterol levels in pigs can be prevented by introduction of a *L. acidophilus* strain that is bile resistant and assimilates cholesterol. These findings were supported by research conducted by Pereira and Gibson (2002a; b) who demonstrated that probiotic strains were able to assimilate cholesterol in the presence of bile into their cellular membranes. Results however, were influenced greatly by the bacterial growth stage and inoculum used as resting cells did not interact with cholesterol as also shown by studies conducted by Dambekodi and Gilliland (1998). St-Onge *et al*. (2000) extensively reviewed the extensive existing studies from animal and human studies which detected moderate cholesterol-lowering was due to consumption of fermented products containing probiotic bacteria. Studies by Gopal *et al*. (1996) also showed cholesterol removal by *Bifidobacterium* spp. and *Lactobacillus acidophilus*.

1.5.2.9. Production of hormones and other agents

The possibility of genetically engineering strains of bacteria that can produce substances such as insulin, androgens, estrogens, growth hormone or cholesterol-lowering compounds, just to mention, a few is intriguing. The ability to produce *in situ* over a long period of time drugs or hormones that are constantly required by individuals suffering from various diseases (i.e. diabetes and hypercholesteremia) is of particular interest. These are problems to this approach, however; e.g. control of production and contamination of normal individuals with the organism. Establishing the maximum achievable production level of the organism in the gut and thereby setting an upper limit on dose may solve the first problem. The contamination problem may be more difficult to solve, although antibiotic sensitivity can be introduced into the strains, so that the organism could be rapidly eliminated if a normal individual is infected with a specifically

designed probiotic. This idea may have too many regulatory problems associated with it; however, it is still something that may have potential use in human disease regulation.

1.5.2.10. Colonisation resistance

The indigenous microflora on body surfaces inhibit the colonisation of non-indigenous microorganisms. Nevertheless, in some cases (potential) pathogenic microorganisms are able to penetrate and/or colonise these body surfaces, due to a massive attack of the pathogens or to a (temporarily) reduced colonisation resistance. In different studies on humans and animals beneficial microorganisms are used to improve the colonisation resistance on body surfaces, such as gastrointestinal-, the urogenital-, and the respiratory-tract.

1.5.3. Application of probiotics

1.5.3.1. Importance of probiotic consumption in humans

The number of food and other dietary adjuncts products containing live *Bifidobacterium* and *Lactobacillus* bacteria have significantly increased over the last 20 years due in part to the beneficial effects these probiotic organisms are believed to provide (Laroia and Martin, 1990). Presented in Table 1.16. is a listing of bacterial species used as probiotic cultures in food products.

Table 1.16. Bacterial species primarily used as probiotic cultures (Krishnakumar and Gordon, 2001)

Species	Strains
Lactobacillus acidophilus	La2, La5 (also known as La1), Johnsonii (La1; also known as Lj1), NCFM, DDS-1, SBT-2062
L. bulgaricus	Lb12
L. lactis	La1
L. plantarum	299v, Lp01
L. rhamnosus	GG, GR-1, 271, LB21
L. reuteri	SD2112 (also known as MM2)
L. casei	Shirota, Immunitass, 744, 01
L. fermentum	RC-14
Bifidobacterium longum	BB536, SBT-2928
B. breve	Yakult
B. bifidum	Bb-12
B. esselnsis	Danone, (Bio Activia)
B. lactis	Bb-02
B. infantis	Shirota, Immunitass, 744, 01

Although research is ongoing, the available evidence indicates that ingestion of probiotic bacteria may promote desirable changes in the gastrointestinal tract of humans (Kaplan and Hutkins, 2000). Table 1.17 describes several probiotics that are utilised in the food and agricultural industry.

Table 1.17. Organisms used as probiotics in the food and agricultural industry (Goldin and Gorbach, 1992)

Organism	Comment
Saccharomyces boulardii	Non-pathogenic yeast used for treatment of diarrhoea
Lactobacillus acidophilus	As a supplement in dairy products and used for fermentations; numerous health claims
L. plantarum	In dairy products, pickled vegetables and silage
Lactobacillus GG	In yoghurt and whey drink; numerous health claims
L. casei subsp. *rhamnosus*	In dairy products and silage
L. brevis	In dairy products and silage
L. delbrueckii spp. bulgaricus	Production of yoghurt; health claims have been made
Streptococcus thermophilus	For the production of yoghurt
Bifidobacterium bifidum	Component of new dairy products and in preparation for new born; health claims
Bifidobacterium infantis	Similar to *B. bifidum*
Enterococcus faecium	Being introduced in certain health products; health claims
L. lactis ssp. *lactis and cremoris*	Used in production of buttermilk and certain cheeses

1.6. Prebiotics

There is currently much interest in the concept of actively improving the host health by managing the colonic microflora. Traditionally, this has been attempted by using probiotics. An alternative approach is the consumption of food ingredients known as prebiotics (Rycroft *et al*., 2001). Prebiotics, as currently conceived of, are all carbohydrates of relatively short chain length (Cummings *et al*., 2001), additionally carbohydrates that have escaped digestion in the upper gastrointestinal tract form the predominant substrates for bacterial growth in the colon (Roberfroid *et al*., 1998a). Present evidence concerning the two most studied prebiotics, fructooligosaccharides and inulin, is consistent with their resisting digestion by gastric juice and pancreatic enzymes *in vivo*. In the large intestine, prebiotics, in addition to their selective effects on bifidobacteria and lactobacilli, influence many aspects of bowel function through fermentation (Alles, 1998; Campbell *et al*., 1997). Short-chain fatty acids are a major product of prebiotic breakdown, but as yet, no characteristic pattern of fermentation has been identified. Through stimulation of bacterial growth and fermentation, prebiotics affect bowel habit and are mildly laxative (Cummings *et al*., 2001).

1.6.1 Definition of prebiotics

1.6.1.1. Definition and concept of prebiotics

The term `prebiotic' was first coined by Gibson and Roberfroid (1995). Prebiotic is defined as `a non digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, that can improve the host health'. The function of prebiotics is to basically stimulate existing metabolisms in the colon (Coussement, 1996). Thus, the prebiotic approach advocates administration of non-viable entities and therefore overcomes survival problems in the upper gastrointestinal tract. The

prebiotic concept considers that many potentially health-promoting micro-organisms, such as bifidobacteria and lactobacilli, are already resident in the human colon. To be an effective prebiotic an ingredient must:

- Neither be hydrolysed nor absorbed in the upper part of the gastrointestinal tract;
- Have a selective fermentation such that the composition of the large intestinal microbiota is altered towards a healthier composition;
- Prebiotics may have many advantages over probiotics. This is firstly related to survivability problems.

These include:

- Maintenance of viability in the product (which, for obvious reasons, will usually be stored under conditions adverse to bacterial growth);
- Gastric acidity;
- Bile salts;
- Pancreatic enzymes and proteins;
- Competition for colonisation sites and nutrients with the resident gastrointestinal flora.

1.6.1.2. Definition of nondigestible oligosaccharides (NDO)

Oligosaccharides are carbohydrates with a low degree of polymersiation (DP) and therefore low molecular weight. They have been variously defined as including anything from 2 to 20 monosaccharide units. The main categories of nondigestible oligosaccharide presently available or in development as food ingredients include carbohydrates in which the monosaccharide unit is fructose, galactose, glucose, and/or xylose. NDOs are readily water soluble and exhibit some sweetness, but solubility decreases with longer chain length. Furthermore due to being undigested in the colon, they have caloric value but due to colonic

fermentation, they have an energy contribution to food of about 1.5 kcal/g, similar to soluble fibre (Roberfroid and Slavin, 2000). Presented in Table 1.18 is a selection of commonly available oligosaccharides, their properties and annual production.

Table 1.18. Commonly available oligosaccharides and other bifidogenic properties (Jelen & Lutz, 1998).

Source	**Product**	**Estimated Annual Production (t)**
Lactose (milk)	Lactulose	20,000
	Lactosucrose	1,600
	Lactitol	n/a
Chicory, Jerusalem artichoke	Fructo-oligosaccharides	12,000
Starch	Malto- and isomalto-oligosaccharides	21,000
Soybean	Raffinose, stachyose	2,000

1.6.2. Description of common prebiotics

1.6.2.1. Oligosaccharides

Interest in fructooligosaccharides as a health-promoting food component is increasing. Oligosaccharides are a group of short chain nondigestible polysaccharides consisting of between approximately 2 and 20 saccharide units, may be linear or branched, and occur in a wide variety of foods (Shin *et al*., 2000). Fructo-oligosaccharides are widely distributed in plants such as onion, leek, asparagus, chicory, Jerusalem artichoke, garlic, wheat and oat as well as soybean asparagus and also made by the action of fructosyltransferase on sucrose. They are not hydrolysed by the human digestive enzymes, but are utilised by intestinal bacteria such as bifidobacteria (Kaplan and Hutkins, 2000; Hidaka *et al*., 1986), the *Bacteroides fragilis* group, peptostreptococcaceae and klebsiellae.

Oligosaccharides can be commercially produced through the hydrolysis of polysaccharides (e.g. dietary fibres, starch) or through enzymatic transfer reactions from lower

molecular weight sugars. An array of oligosaccharides has been tested using various *in vitro* methods, animal models and human clinical trials for their prebiotics effect on probiotic bacteria. Significant increases of bifidobacteria populations in faeces after consumption of fructo-oligosacccharides after relatively short periods of time hav been reported (Mitsouka *et al.,* 1987).

1.6.2.2. Inulin

Inulin is a blend of fructan chains found widely distributed in nature as plant storage carbohydrates (Wang and Gibson, 1993), and is present in more than 36,000 plant species. The majority of inulin commercially available today is extracted from chicory roots. Chemically, inulin is a polydisperse β-(2,1) fructan. The fructose units in the mixture of linear fructose polymers and oligomers are each linked by β-(2,1) bonds. A glucose molecule typically resides at the end of each fructose chain and is linked by an α-(1,2) bond, similar to sucrose. Chain lengths of these chicory fructans range from 2-60, with an average degree of polymerisation of 10 (Flickinger *et al*., 2003). *In vitro* they selectively stimulate the growth of *Bifidobacterium* (Gibson *et al*., 1995).

1.6.2.3. Isomalto-oligosaccharides

Isomalto-oligosaccharides exist in fermented foods such as misio, soy, sauce, sake and honey. The effect of isomalto-oligosaccharides on human faecal flora was also studied. Bifidobacteria and the *Bacteroides fragilis* group were able to utilise isomalto-oligosaccharides, but *Escherichia coli* and other bacteria were not. After the administration of 13.5g of isomalto-oligosaccharides per day for 2 weeks to healthy adults, bifidobacteria remarkably increased (Kohmoto *et al*., 1988).

1.6.2.4. Lactulose

Lactulose is a synthetic disaccharide in the form Gal β1-4 fru. Lactulose has been used as a laxative as it is not hydrolysed or absorbed in the small intestine. However, at sub-laxative doses lactulose has received attention as a bifidogenic factor and has been administered as such (Tamura, 1983; Modler *et al*., 1990; Modler, 1993; Kiyosawa, 1986). *In vitro*, lactulose increased lactobacilli and bifidobacteria and significantly decreased bacteroides in mixed continuous faecal culture. The feeding of lactulose to rats significantly increased bifidobacteria; however, only a limited number of bacterial groups was enumerated (Suzuki *et al*., 1985).

In a human trial, bifidobacteria significantly increased while clostridia, bacteroides, streptococci and Enterobacteriaceae decreased on the feeding of 3 g/d lactulose to eight volunteers (five male, three female) for 14 days (Terada *et al*., 1992). Small decreases in bacteroides and lactobacilli during the test period were also determined. In addition, decreases in the detrimental metabolites ammonia, indole, phenol, *p*-cresol and skatole, and enzymes β-glucuronidase, nitroreductase and azoreductase supported beneficial claims of lactulose.

Bifidobacterium longum has been shown to afford protection against colon tumourigenesis. Lactulose (4-O-β-D-galactopyranosyl-D-fructose), a keto analogue of lactose, serves as a substrate for preferential growth and increased development of *Bifidobacterium*). Lactulose is not metabolized by human or animal species and resists degradation by the lactases in the digestive tract. Lactoferrin and its three metal complexes (Fe, Cu, Zn) have a promoting effect on the growth of eight species of *Bifidobacterium*, five of human origin and three of animal origin, at the beginning of the logarithmic growth phase. Furthermore, these lactoferrin-metal complexes demonstrate an anti-bacterial activity versus *E. coli* and *Staphylococcus aureus* (Salminen *et al*., 1993). These particular oligosaccharides seem to be preferentially utilised by

bifidobacteria in the large intestine causing elevated growth of this bacterial genus (Gibson *et al*., 1994).

1.6.2.5. Fructo-oligosaccharides (FOS)

Inulin and oligofructose have a specific chemical structure which our digestive enzymes cannot hydrolyse. Both substances are metabolised as dietary fibres in our body. Inulin and oligofructose also show beneficial dietary fibre effects such as a relief of constipation, increased stool volume and an increased faecal acidity (Coussement, 1996). Inulin and oligofructose belong to a group of carbohydrates known as non-digestible oligosaccharides (NDO), which are commonly consumed in a standard Western diet (Gibson *et al*., 1994).

Chemically speaking, inulin is a mixture of poly- and oligo-saccharides of which almost all have the chemical structure GF_n (G = glucose, F = fructose and n = number of fructose units linked to one another). The maximum amount of fructoses in inulin from chicory is about 60. The links between the molecules are of a very special type: the β(2-1) form, which makes these molecules indigestible for all higher animals.

Inulin-type fructans contain both GF_n (α D glucosyl- [β D fructosyl]$_{n-1}$-D fructoside) and FF_n (β D fructosyl-[β D fructosyl]$_{n-1}$-D fructoside) molecules, with the number of fructose units varying from two to more than 70 units. The structural relatives of inulin, fructo-oligosaccharides (FOS, a lower molecular weight version) are well documented oligosaccharides with regard to their effect on intestinal bifidobacteria and are considered important prebiotics. Inulin naturally occurs in thousands of different plants with garlic, onion, asparagus, chicory, artichoke, wheat and leak being especially rich.

The two different types of fructo-oligosaccharides are common. Firstly, inulin extracted from chicory roots can be hydrolysed under controlled conditions by the enzyme inulinase to

produce short-chain FOS represented as Glu-α1-2[β-D-Fru 1-2]^{n} where n = 2-9. Another FOS product known as `neosugar' or `meioligo' is a mixture of three oligosaccharides of different lengths, i.e. 1-ketose (Glu-Fru$_2$) and 1^{F}-β-fructosylnystose (Glu-Fru^{4}). The mixture is enzymatically synthesised from sucrose by the transfructosylation action of β-fructosidase from the fungus *Aspergillus niger*.

It is accepted that FOS are not degraded or absorbed in the upper human gastrointestinal tract. As such, they enter the colon intact where they are susceptible to metabolism by the resident microbiota (Hidaka *et al*., 1991). The β configuration of anomeric C_2 in fructose monomers, is thought to make FOS resistant to hydrolysis by human digestive enzymes which display a high degree of specificity for glycosidic linkages (Gibson *et al*., 2000)

In pure culture, most species of bifidobacteria are adept at the utilisation of inulin-type fructans. Many other bacteria are also capable of metabolising these substrates including *Klebsiella pneumoniae, Staphylococcus aureus* and *S. epidermis*, *Enterococcus faecalis* and *E. faecium, Bacteroides vulgatus, B. thetaiotaomicron, B. ovatus* and *B. fragilis, Lactobacillus acidophilus* and *Clostridium* sp. In mixed batch and chemostat culture studies, it has been demonstrated that both inulin and its hydrolysate selectively stimulated the growth of bifidobacteria, which, at the end of the incubation period, become numerically predominant.

Batch culture studies where faecal slurries were incubated with FOS, starch, polydextrose, fructose and pectin for 12 hours (Wang and Gibson, 1993) showed the greatest increase in bifidobacteria with the FOS, indicating the prebiotic nature of these substrates. Continuous culture systems inoculated with faecal slurries were later used to investigate the fermentation. In accordance with earlier studies, bifidobacteria and to a lesser extent lactobacilli preferred FOS to

glucose. Three-stage chemostats (gut models) confirmed an enhanced proliferation of bifidobacteria by FOS in conditions resembling the proximal colon.

1.6.2.6. Galacto-oligosaccharides

Galacto-oligosaccharides are galactose-containing oligosaccharides of the form Glu α1-4[β Gal 1-6]$_n$ where n = 2 to 5, and are produced from lactose syrup using the transgalactosylase activity of the enzyme β-galactosidase. Studies by Taneka *et al.* (1983) showed good growth of all eight bifidobacteria strains tested, TOS was concluded to be a suitable bifidobacterial-promoting substrate.

1.6.2.7. Soybean oligosaccharides

The predominant oligosaccharides in soybeans are the trisaccharide raffinose and the tetrasaccharide stachyose which are able to reach the colon and are thought to stimulate bifidobacteria. In pure culture studies, soybean oligosaccharides were fermented to a far greater degree by bifidobacteria than any other organisms tested. Furthermore, the addition of a low concentration of soybean oligosaccharides to a two stage continuous culture of faecal bacteria resulted in a threefold increase in the proportion of bifidobacteria in the total bacterial count (Gibson *et al*., 2001).

1.6.2.8. Lactosucrose

Lactosucrose is produced from a mixture of lactose and sucrose using the enzyme β-fructosidase and has been found to be bifidogenic in pure culture studies (Tamura, 1983). A later pure culture study compared lactosucrose with lactulose, FOS, soybean oligosaccharides, raffinose and glucose for its utilisation by various intestinal bacteria (Hara *et al*., 1994). Six bifidobacteria and three lactobacilli strains grew to the same extent (comparable end pH) on lactosucrose and glucose, whereas all the other organisms tested preferred glucose.

1.6.3. Utilisation of prebiotics by consumers

Japan is at the forefront in the development and use of prebiotics due in principal to the influence of the food industry regulatory system. This allows some degree of health claim for a product. The permitted health claims are rather vague stating improvements in well-being and also carry warnings. Prebiotics have been incorporated into many functional foods and drinks in Japan. These products include; soft drinks, candies, biscuit, frozen yoghurt, table-top sweetener, lactic acid bacteria drink, coffer drink and custard desserts. The oligosaccharides used in the application of these `foods for specified health use' (FOSHU) in Japan include: fructo-oligosaccharides, lactulose, lactosucrose, xylo-oligosaccharides, isomalto-oligosaccharides, soybean oligosaccharides and transgalacto-oligosaccharides.

The incorporation of prebiotic into foodstuffs in Europe is significantly less advanced than in Japan. However, the potential market of a dietary ingredient with a carbohydrate base is enormous. This includes yoghurts, cereals, confectionary, biscuits, cakes, sauces, powdered drinks, pasta, snack foods, processed vegetables, rice, cereal bars, breads, infant formula foods and fruit juices amongst others. Specific examples of prebiotic containing foods that already exist include *Frutex, Actilife, Symbalance, Aviva, LactoPro+, b2* and *Kinder-Flakes*. Although, these particular products are based on fructo-oligosaccharides, new biotechnological capabilities promise to expand the prebiotic market exponentially. In addition, the main European manufacturers of prebiotics (e.g. Sensus, Orafti and Solvay) all produce prebiotic supplements to be consumed on a daily basis.

1.6.4. Fermentation of inulin

Inulin-type fructans resist digestion in the upper part of the gastrointestinal tract. Furthermore, there is no evidence that they are absorbed to any significant extent within the

gastrointestinal tract. Therefore, it has been proposed that inulin-type fructans be classified as ‘colonic food,’ i.e. a `food entering the colon and serving substrate for the endogenous bacteria, thus indirectly providing the host with energy and metabolic substrates’ (Gibson and Roberfroid, 1995).

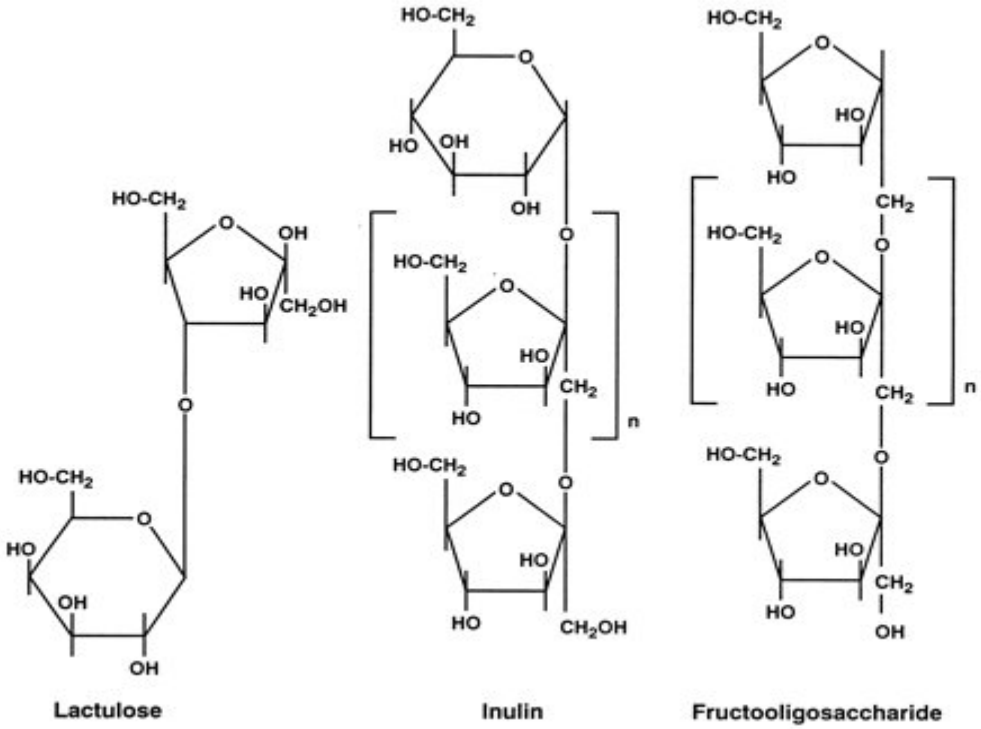

Figure 1.2. Chemical structures of common prebiotic products (Gibson and Roberfroid, 1995a)

1.6.4.1. Fermentation in the large bowel

The large bowel is the most highly colonised section of the gastrointestinal tract containing approximately 10^{12} bacteria for every gram of gut contents. Colonic bacteria which are mainly anaerobes produce a wide variety of compounds during the process of fermentation. The fermentation of carbohydrates produce short-chain carboxylic acids (mainly acetate, propionate, and butyrate) and lactate, which allows the host to salvage part of the energy of nondigestible carbohydrates. Evidence that bacteria colonising the large bowel ferment inulin-

type fructans is supported by a large number of *in vitro* and *in vivo* studies, which, in addition, confirm the production of lactic and short-chain carboxylic acids as end products of fermentation. In addition, it has been repeatedly demonstrated that in human *in vivo* studies, this fermentation leads to the selective growth of bifidobacteria (Roberforid and Delzenne, 1998).

In vitro data demonstrate several facts: that inulin-type fructans are well fermented by human faecal slurries; that in pure cultures and among the bacteria known to be present in human feces, bifidobacteria among several other genus metabolise these carbohydrates; and that in mixed cultures, which replicate bacterial conditions similar to those in the large bowel, the growth of bifidobacteria is selectively stimulated to such an extent that these bacteria become largely predominant over the other populations (Roberfroid and Delzenne, 1998).

1.6.4.2. In vitro fermentation of inulin-type fructans

Evidence that inulin-type fructans are fermented by the colonic microbiota is the demonstration that these carbohydrates are metabolised when incubated with human fecal slurries in anaerobic batch cultures. During fermentation, production of various acids leads to changes in culture pH are convincing evidence as well as with pure cultures an easy method to identify which bacteria have the potential to perform such a metabolic process. Furthermore, by estimating the size of the drop in culture pH over a given period of incubation, it is possible to compare different substrates on a semi quantitative basis.

In summary, both *in vitro* and *in vivo* studies on the fermentation of inulin-type fructans demonstrate that they are metabolised by anaerobic bacteria that are normal constituents of the colonic microbiota. But even if in pure cultures miscellaneous bacteria species have the capacity to use inulin-type fructans as a fermentation substrate, in mixed cultures replicating the large bowel as well as *in vivo* in human volunteers, these fructans have convincingly been shown to selectively stimulate the growth of bifidobacteria.

1.6.6. Consumption of prebiotics by humans

The majority of reported trials carried out on the prebiotic effects of oligosaccharides are positive, however, the scientific control of these trials can be variable. Presented in Table 1.19 are a few examples of volunteer prebiotic human feeding trials carried out worldwide over the last two decades. Number of subjects, dosage consumed, substrate used, length of study and outcomes are presented.

Table 1.19. Examples of human studies with oligosaccharides (Gibson *et al*., 2000)

Substrate	Subjects	Dose & Duration	Results	Reference
FOS	10 adults	12.5 g/d for 12 d	Significant increase in bifidobacteria	Bouhnik *et al*. (1996)
Inulin	10 senile adults	20 g/d. for 8 d, then 40 g/d for 11 d	Significant increase in bifidobacteria	Kleesen *et al*. (1997)
Neosugar	23 senile adults	8 g/d for 14 d	Significant increase in bifidobacteria	Hidaka *et al*. (1986)
TOS	8 adults	10 g/d for 21 d	Significant increased bifidobacteria; significant increase in breath H_2	Bouhnik *et al*. (1997)
Raffinose	7 adults	15 g/d for 4 wk	Significant increase in bifidobacteria, significant decrease in bacteroides and clostridia	Benno *et al*. (1987)
Lactulose	12 adults	2 x 10 g/d for 4 wk	Bifidobacteria, streptococci and lactobacilli significantly increased.	Ballongue *et al*. (1997)
XOS	5 men	20 g/d for 3 wk	Significantly increased bifidobacteria, decrease in bacteroides	Okazaki *et al*. (1990)
SOE	6 adults	10 g/d for 3 wk	Significant increase in bifidobacteria and lactobacilli; large decrease in clostridia and peptostreptococci	Hayakawa *et al*. (1990)
Lacto-sucrose (55%)	13 senile adults	10.3 g/d for 3 wk	Significant increase in bifidobacteria	Kumemura *et al*. (1992)
IMO	9 men	10 g/d for 2 wk	Significantly increased bifidobacteria	Kohmoto *et al*. (1991)

1.7. Clinical evaluation techniques for consumption of probiotic bacteria and prebiotics

1.7.1. Intestinal content sampling techniques

1.7.1.1. Testing for functionality: how to determine prebiotics effects

For prebiotics, a number of volunteer trials have already been carried out, with positive effects. However, it is imperative that the best technologies available are used in conjugation with well-controlled trials.

The easiest way to determine how bacterial substrates are metabolised is the challenge the test material with pure cultures of selected microorganisms. The substrate would be added to a basal growth medium and bacterial growth determined during a time course incubation. Even so, for gut micro-organisms, the approach does involve something of a challenge in that anaerobic growth conditions must be induced along with standard microbiological aseptic techniques. The usual way to monitor the growth response is by measuring the optical density of the culture or plate count for viable colonies. The comparison would be with bacterial growth in the absence of test material.

This approach gives a reasonable comparative assessment of metabolism in monoculture, but does not induce any element of competition. The problem is that the gut microbiota has at least 500 described species, while latest estimates are that over 1000 different microbial types may exist. Thus, the approach cannot identify true selectivity and therefore the prebiotics activity of a particular substrate.

A more refined approach is to use mixed culture experiments with selected gut microbial species. This introduces some element of competition between the microorganisms, but again does not adequately resemble the complex interactions that occur in the human gut microflora.

However, the approach is very useful for predicting how the large micro-organisms for prebiotics intake may affect potentially pathogenic species.

1.7.1.2. Mixed cultural bacterial fermenters

A common approach towards the determination of gut microbial activities is to use batch culture fermenters inoculated with faecal bacteria. These would be kept anaerobic by the infusion of oxygen-free gas like nitrogen, have pH control and be continuously mixed. However, these are closed systems where the substrate is limited, so are only appropriate for time-course experiments. The recognised bacterial dynamics, of standard growth curves, would dictate that the substrate supply would diminish and cause `stationary' culture response. In contrast, the human situation is that the colon is constantly replenished with microbial nutrients such that the ecosystem is frequently charged with growth substrates.

A more physiologically relevant approach is therefore continuous culture, whereby a constant input of nutrients may be supplied and other physiological parameters like varying dilution rate can also be imposed. Semi-continuous culture is one variable whereby `pulse-type' experiments can be carried out where the medium is added and spent culture removed at specific intervals. The most conventional approach is the chemostat where a continuous supply of growth medium is fed to the culture. Usually, one substrate is given in growth-limiting quantities such that enrichment occurs whereby bacteria that can respond to the limited nutrient will eventually predominate in the system. This is an effective way to determine the metabolism of candidate prebiotics. The drawback is that the one-stage continuous culture chemostat is a homogenous system and varying physiochemical determinants cannot be imposed, while culture results obtained in these studies compose between 50% and 80% of the total microscopic count (Tannock, 2001).

1.7.1.3. In vitro gut models

In the proximal region, there is a very ready supply of substrate. Hence, bacteria grow quickly and the pH is acidic (due to the formation of acidic end products). In the left side bacteria grow more slowly, the nutrient supply is diminished and it is environmentally more pH neutral. As such, an efficient gut model system would minimise these different physiochemical parameters.

One model is the three-phase chemostat based on gut model simulation in different anatomical areas of the large gut, such as the right, transverse and left sides. This system has been validated against samples taken at autopsy and gives a very close approximation to fermentative bacterial events that occur *in situ*. Studies on the development of the microbial flora in the three vessels can be performed such that fermentation characteristics in the varying areas may be predicted. The system consists of three vessels, of increasing size, aligned in series such that a sequential feeding of growth medium occurs. The vessels are pH regulated to reflect *in vivo* differences. As such, vessel 1 has a high availability of substrate; a bacterium grows quickly and is operated at an acidic pH, similar to events in the proximal colon. In contrast, the final vessel resembles the neutral pH, slow bacterial rate and low substrate availability, which is characteristic of distal regions. After inoculation with faeces, an equilibration period is allowed such that the bacterial profiles respond to their imposed conditions. Such gut models have been applied to prebiotic research and gives very effective data. Future developments in chemical engineering, the use of intestinal cell tissues in such models and dialysis systems will help improve the situation whereby biofilm communities can also be predicted.

1.7.1.4. Animal models

Animals, often rats or mice, have been used to determine the prebiotic nature of a substrate. Conventional, gnotobiotic (germ-free) rats or those inoculated with one or a limited

number of micro-organisms may be used to investigate prebiotic interactions, although this does not resemble the usual situation in the gut. Rats may be associated with a human faecal flora, known as HFA rats, and give a further representation of the situation in the human intestine, although the intestinal physiology is not the same. A major drawback with laboratory animal experiments is the differing (gut) anatomy as well as coprophagy. A more realistic model of the human gut would be to use primates; however, there are clear logistical and expense problems with this approach.

1.7.1.5. Human trials

The definitive assessment of prebiotic effect is to feed candidate substrates or food to human volunteers and assess microbiological changes in stools. As faeces are the only readily accessible area of gut contents, it is difficult to predict fermentation in more proximal gut contents. However, if a prebiotic can induce faecal changes in the microbiota, then it may be assumed that more saccharolytic environments (e.g. the right side of the large gut) have also been affected.

One major drawback towards microbial assessments on faeces is that conventional bacteriology dictates that freshly voided samples are processed. This is because gut bacteria react differently to storage, thus grossly affecting phenotypic (characterisation) traits. In this case, new discriminatory techniques that involve a molecular biological approach towards bacterial characterisation are of enormous value. Such technology is highly reliable and allows the processing of frozen specimens and therefore multiple centre trials.

Human trials may be carried out on volunteers who are on controlled diets, or are free living. The former is difficult to control, although the use of radio-opaque markers in the diet and their detection in stools is a useful measure of gut transit time and also compliance. Moreover, the trials should be carried out in a double-blind manner with good placebo control. In fact, some

studies may be `triple blind' in that not only the investigators and volunteers are unaware of the feeding form, but also those carrying out any statistical analyses. In this way, a candidate prebiotic could be fed and bacterial changes determined. Two types of challenges are possible. One is a crossover trial whereby volunteers would ingest both the test and placebo material (blind coded) for specific periods. Another form is the parallel study whereby volunteers would be randomly categorised into placebo or test material groups. Presented in Table 1.20 is a listing of strains of probiotic bacteria used in commercial production and their clinical evidence in human testing.

Table 1.20. Strains of probiotic bacteria in decreasing order of clinical evidence (Playne, 2002)

Strain	**Conditions**
Lactobacillus rhamnous GG (Valio)	1, 2, 4, 5, 6, 7, 8, (12), 14, 15
Saccharomyces cerevisiae Boulardii (Biocodex)	2, 3, 4, 5, 7, 11
Lactobacillus paracasei Shirota (Yakult)	2, 5, 6, 9, (10), 11, (12), 15
Bifidobacterium lactis BB12 (Chr Hansen)	1, 2, 3, 4, 5, 6, 11, 15
Lactobacillus reuteri (Biogaia)	1, 5, (10), (12)
Lactobacillus johnsonii La1 (Nestle)	6, 11, 14, 15
Enterococcus faecium SF68 (Cernelle)	2, 5, 10, (12), 13
Lactobacillus acidophilus La5 (Chr Hansen)*	2, 4, 5, 6, 11
Bifidobacterium longum BB536 (Morinaga)	2, 5?, 11, (12), (15)
Bifidobacterium breve (Yakult)	(1), 5
Lactobacillus acidophilus NCFM (Rhodia USA)	1?, 5, 6, (12)
Lactobacillus plantarum 299v (ProViva, Sweden)	5, 13

Condition: 1=rotaviral diarrhoea; 2=antibiotic-associated diarrhoea; 3=*Clostridium difficule* pseudomembranous colitis; 4=traveller's diarrhoea; 5=other acute bacterial diarrhoea; 6=lactose intolerance; 7=bacterial vaginitis; 8=atopic eczema and food allergy; 9=bladder cancer; 10=cholesterol; 11 chronic constipation; 12=bowel cancer; 13=irritable bowel syndrome; 14=*Helicobacter pylori*; 15=immune response modulation.
?=doubtful evidence; ( )=animal data and/or biomarkers only
* Data for this strain uncertain, as it was co-administered with *B. lactis* BB12 usually.

1.7.1.6. A molecular approach to bacterial characterisation

A critical aspect of prebiotic assessment, whether *in vitro* or *in vivo* models are used, is a reliable determination of the bacteriological aspects. The evaluation of the prebiotic potential of the oligosaccharides has, to date, been mostly performed using conventional microbiological techniques. Gut microbiology is usually carried out by plating faecal micro-organisms onto selective agars designed to recover the numerically predominant groups of bacteria. However, the agars used are only semi-selective, do not recover non-culturable bacteria (which may represent over 50% of the overall diversity) and allow operator subjectivity in terms of microbial characterisation, which is usually based on limited phenotypic procedures. As such, if the approach is to be use then a vast array of selective agars should be involved with intensive characterisation of the colonies which develop. This may involve:

- Morphological description of colonies that develop (shape, margin, consistency, pigments and extracellular secretions;
- Microscopic characteristics (size of cells, nature of growth, shape, spores);
- Gram reaction (and possibly other staining techniques such as acid fast reaction);
- Biochemical traits (e.g. the use of analytical profile indices, analysis of fermentations end products which involves growing the colonies in a liquid growth medium and subsequent analysis);
- The assay of specific enzymes (e.g. fructosidase activities in bifidobacteria);
- Other biomarkers (such as specific cell wall antigens, cellular fatty acids, plasmid profiles, antibiotic sensitivities).

1.7.1.7. Genotypic characterisations

Many studies have reported the results of sampling small subunit ribosomal DNA (rDNA) from human faecal samples (Matsuki, 1999, 1998; Wilson and Blitchington, 1996). The genotypic identity of gut bacteria can be investigated by PCR-16S-rRNA gene restriction fragment length polymorphism (RFLP) and partial gene sequence analysis. 6S rRNA consists of a mosaic of universal, semi-conserved and non-conserved regions and enables the molecule to span great, as well as to measure close, genetic relationships. The `sequences' of the hypervariable regions of the molecule are characteristic of different organisms and to provide a rapid and reproducible means of determining genotype. Full 16S rRNA genes can be amplified from single colonies utilising primers to conserved regions proximal to the 5' to 3' termini of the gene. rDNA products are subjected to RFLP analysis (restriction with endonuclease and electrophorectic analysis), and each bacterial type is characterised by a simple, but highly specific, series of rDNA restriction patterns.

1.7.1.8. Culturing bacteria

Most aerobes and facultative anaerobes can be cultured relatively easily on commercially available highly selective media. Although these are inhibitory and even apparently reduce the overall count of the organisms for which they are selective they do at least allow the partial enumeration of easily recognised groups such as `coliforms', staphylococci, streptococci, yeast and lactobacilli. Few media of this sort are available for strict anaerobes. Almost all the bacteria that grow in the intestine are non-sporulating anaerobes. Thus, *Escherichia coli*, usually thought of as a typical faecal organism, constitute less than 1% of the bacteria in faeces. Many of the bacteria are extremely sensitive to oxygen, especially on initial isolation, and thus the usual techniques for the study of anaerobes from clinical sources are not ideal.

The use of prereduced, anaerobically sterilised media or an anaerobic chamber is essential. Serial dilutions of specimens may be prepared in anaerobic dilution blanks, the manipulations being performed in an anaerobic chamber or, if the pre-reduced anaerobically sterilised medium is used, in tubes gassed with oxygen-free gas. Plates or roll tubes of pre-reduced media are inoculated from these dilution series. This enables an estimate to be made of the numbers of bacteria present by counting colonies; however, in faeces and with the contents of the large intestine the situation is complicated by the large number of bacterial species present. It is easy to obtain a total viable count but very laborious to obtain counts of the identifiable bacterial species.

1.7.2. Selection of prebiotics

One of the biggest constraints in the development of prebiotics is the limited knowledge of the structure-function relationships in these molecules. At the current time, there is little information on the relatively efficacy and selectivity of these molecules. A useful prebiotic would:

- Have low dosage forms;
- Be non-carcinogenic;
- Have a low calorific value;
- Have multifunctional properties;
- Easily incorporated into food vehicles;
- Exert good preservative and drying characteristics;
- Target the distal colon.

1.8. *Helicobacter pylori*

1.8.1 History of Helicobacter pylori

Helicobacter pylori is a Gram negative, spiral-shaped, microaerophilic organism, that colonizes the mucosal layer of the gastric epithelium. The morphology switches from the spiral form, possibly through a U form, into a non-dividing coccoid form during various environmental conditions, including aerobiosis, temperature changes, extended incubation and antibiotic treatment (Nilsson *et al.*, 2002). Chronic infection with *H. pylori* has been identified as the major etiological factor in gastritis, gastric ulcers, gastric atrophy, and gastric carcinoma (Van de bovenkamp *et al*., 2003). In 1994, the International Agency for Cancer Research, an arm of the World Health Organization, reviewed the evidence and classified *H. pylori* as a human carcinogen (Dunn *et al*., 1997).

H. pylori was first isolated by Marshall and Warren in 1982 and the association of the presence of this organism with histologic gastritis was established (Lee, 1991). Infection with *H. pylori* is now estimated to be 40-70% worldwide (Benson *et al*., 2004). Most infections are acquired in childhood and most studies have indicated that males and females are infected at approximately the same rate. Three routes of transmission from the stomach of an infected individual (the stomach being the main reservoir of *H. pylori*) to another have been described. These include (i) the iatrogenic route (via tubes or endoscopes), which is least common, (ii) faecal-oral transmission (the most common) and (iii) the oral-oral transmission, which has been identified in the case of African women who pre-masticate foods given to their infants. The organism has not been isolated form water, and its isolation in faecal matter is not common. There has been no identified association of the infection with sexual transmission (Dunn *et al*., 1997).

1.8.1.1 Pathogenicity of Helicobacter pylori

The pathogenicity of the organisms may be attributed to two sets of determinants: (a) virulence factors such as urease, vacuolating cytotoxin, Interleukin-8; and (b) maintenance factors such as motility, bacterial adhesins and adaptive enzymes. *H. pylori* colonize and survive in a very hostile environment. Several tests are available to diagnose *H. pylori* infection, including histological examination of gastric tissue, bacterial culture, ^{13}C-urea breath tests, PCR analysis or gastric juice PCR, serology and urinary excretion of (^{15}N)ammonia. In the United States and Europe, about 60% of all *H. pylori* strains possess a gene called *cagA*, which is a marker for the cag region, a pathogenicity island of about 35 kb. Infection with *cagA*$^{+}$ strains enhances the risk for development of duodenal ulcers and adenocarcinoma of the distal stomach. Essentially all *H. pylori* strains also carry a gene called *vacA* which in certain strains allows the in vitro expression of a protein cytotoxin that induces vacuolation in a wide variety of eukaryotic cells (Dunn *et al*., 1997). No close homologs are known for the *cagA* or *vacA* in other *Helicobacter* species or in other bacteria, suggesting that they reflect a human gastric-specific gene. *H. pylori* induced inflammation affects various cell types in the stomach that are important in acid homeostasis and recently, gastric *H. pylori* colonization has been shown to affect expression of leptin and ghrelin, hormones that control appetite and satiety (Blaser and Atherton, 2004).

1.8.1.2 Traditional treatment of H. pylori

The gold standard for anti- *H. pylori* treatment includes a one-week triple therapy, combining acid suppression with two antibiotics. Triple therapy including clarithromycin, amoxicillin, and a proton pump inhibitor (PPI) is the treatment of choice, while another option is to prescribe tetracycline and metronidazole with the PPI or ranitidine bismuth citrate. This treatment represents the best choice especially in terms of efficacy, tolerability, simplicity of

administration and compliance are considered (European Study Group, 1997). Despite the effective antibiotic therapies available, there are side effects which are a common cause of treatment discontinuation. Another concern is induction of resistance to antibacterial drugs (Sgouras *et al*., 2004). Hence there has been an interest in developing alternative therapies, including use of probiotic organisms.

1.8.2 Probiotics bacteria with prebiotics and H. pylori

Lactic acid bacteria (LAB) are widely used in the production of fermented foods and beverages and contribute both to the sensory qualities of the food and to the prevention of spoilage. Moreover, they are present in large numbers in the normal human and animal gastrointestinal flora (Sgouras *et al*., 2004).

Prebiotics have been used in conjunction with probiotics to aid their survival and action. Several carbohydrate derivatives such as oligosaccharides are known to have a prebiotic action and several have 'bifidogenic' activity when tested under in vitro conditions in a gut model. Oligosaccharides can be designed with specific linkages and different degrees of polymerization which are resistant to digestion by human luminal enzymes (Olano-martin *et al*., 2000). Presently there is an attempt to develop 'synbiotics', i.e. products containing both probiotics and targeted prebiotics as a therapeutic agent for treatment of several gastrointestinal diseases. Although prebiotics have not been speciafically designed for treating *H. pylori*, they are useful in enhancing the chances of survival and the viability of probiotic organisms. There is a possibility to design oligosaccharides that mimic *H. pylori* attachment to the gastric cells, and thus may be used to compete for attachment sites on the gastric membrane.

Health benefits of probiotic organisms include their ability to relieve symptoms of lactose intolerance (De verse *et al*., 1992), immunomodulation, cholesterol lowering potential (Noh *et al*., 1997; Liong and Shah, 2005), antimutagenic activity (Lankaputra and Shah, 1998) and

treatment of diarrhoea (Guandilini *et al*., 2000) to name a few. Many mechanisms have been postulated to explain how probiotics enhance intestinal health, including competition for limited nutrients, inhibition of epithelial and mucosal adherence of pathogens, inhibition of epithelial invasion by pathogens, the production of antimicrobial substances and/or stimulation of mucosal immunity (Servin and Coconnier, 2003). Although probiotics have proven to be a promising treatment, controlled clinical trials are necessary to validate the benefit of probiotics (O'sullivan *et al*., 2005). Probiotics are known to adhere to the intestinal cells and even gastric cells, but often it is not clear whether the probiotic organisms actually colonize the gastrointestinal tract, as they are not recovered from the faeces once the probiotic therapy is discontinued.

1.8.2.1 Effect of probiotics organisms on H. pylori

Several authors have studied the inhibitory effect of probiotic organisms on *H. pylori*, with the aim to develop an alternative treatment to the current antibiotic therapy. Wang *et al.*, (2004) studied the effect of consumption of *Lactobacillus*- and *Bifidobacterium*-containing yoghurt in subjects positive for *H. pylori* infection and concluded that regular intake of yoghurt containing these organisms can effectively suppress *H. pylori* infection in humans. *Lactobacillus acidophilus* (johnsonii) La1 culture supernatant was shown to be inhibitory in vitro and in vivo against *H. pylori* infection in humans (Michetti *at al*., 1999). In vitro inhibitory activity against several strains of *H. pylori* was observed in the presence of viable *L. casei* strain Shirota cells, and a significant reduction in the levels of *H. pylori* colonization was observed in the antrum and body mucosa in vivo when treated with *L. casei* strain Shirota (Sgouras *et al*., 2004). *L. gasseri* OLL2716 in ingested yogurt was determined to be effective in both suppressing *H. pylori* infection in human subjects, as well as reducing gastric mucosal inflammation (Sakamoto *et al*., 2001). Treatment of *H. pylori* by the oral administration of *L. salivarius* in a gnotobiotic murine model was attributed to the high levels of lactic acid produced by the probiotic which mediated

the suppression of colonization (Aiba *et al*., 1998). Armuzzi *et al*., (2001) investigated the effect of administering *L. GG* in conjunction with the standard triple therapy to minimize the associated gastrointestinal side effects, and observed improved treatment tolerability as well as a significant reduction in the side effects experienced. It has been reported that *L. reuteri* strains share glycolipid specificity with *H. pylori* and can therefore inhibit binding of the pathogen to the glycolipid receptors (Mukai *et al*., 2002). The MUC5AC glycoprotein has been identified as the primary receptor for *H. pylori* in the human stomach (Van de bovenkamp *et al*., 2003). *L. reuteri* and *L. paracasei* demonstrated probiotic properties by direct modulation of the mucosal inflammatory response in a *H. hepaticus*- challenged IL-10 deficient murine colitis model (Pena *et al*., 2005).

Tursi *et al.* (2004) studied the effect of 1- day quadruple therapy with *L. casei* subsp. *casei* DG, administered to patients who had failed the first attempt at eradication of *H. pylori*. They reported that probiotic supplementation reduced the side-effects and permitted a slight improvement in eradicating *H. pylori*. Pretreatment of mice with *L. rhamnosus* (R0011) and *L. acidophilus* (R0052) helped reduce the numbers of colonizing *H. pylori* as well as the bacterial-induced inflammation in mice, but did not prevent *H. pylori* induced apoptosis in the gastric mucosa (Johnson-Henry *et al*., 2004). High oral doses of *L. brevis* proved to be ineffective in eradicating *H. pylori* from the stomach of *H. pylori*-positive patients, but did reduce the intragastric bacterial load. The authors concluded that *L. brevis* has an enzyme, arginine deiminase, which may alter the polyamine metabolism leading to a reduction in colonization by *H. pylori* (Linsalata *et al*., 2004). A lyophilized and inactivated culture supplement of *L. acidophilus* increased the eradication rates of a standard triple anti-*H. pylori* therapy in a randomized human trial (Canducci *et al*., 2000). In another study, patients positive for *H. pylori* were fed yogurt containing lactobacilli and bifidobacteria, in conjunction with the regular triple

therapy. Not only were higher eradication rates seen, but there was a restoration of depletion of the bifidobacteria after triple therapy (Sheu *et al*., 2002). Similarly, *L. casei* Shirota, and *L. acidophilus* are able to inhibit the growth of *H. pylori*. In an intervention study, 14 patients infected with *H. pylori* received *L. casei* Shirota (2×10^{10} cfu/day) fermented milk for 6 weeks. *H. pylori* bacterial load was assessed by the breath urea test. Ureolytic activity was reduced in 64% of the patients that consumed fermented products, compared to 33% of the control group (Cats *et al.,* 2003). Recently there has been an attempt to characterize the substance(s) produced by probiotics that is inhibitory to *H. pylori*.

Lactic acid is a well known antimicrobial agent, and works by lowering the pH to a value inhibitory to most microorganisms. It is produced by several lactic acid bacteria and that may account for the inhibitory activity seen. Alakomi *et al*. (2000) elucidated the mechanism of lactic acid on Gram negative organisms, and found that lactic acid disrupts the outer membrane (lipopolysaccharide layer) in these organisms, thus making it susceptible to detergents or lysozyme. Suppression of *H. pylori* by *L. salivarius* has been attributed to high levels of lactic acid produced by the lactobacilli (Sakamoto *et al*., 2001). Other metabolites produced by probiotics, which are known to kill *H. pylori* in vitro include formate and sodium acetate, along with lactic acid (Oh *et al*., 2002). Midolo *et al*. (1995) concluded that the effect of lactic acid on *H. pylori* is dependent on both pH and concentration. They also found other acids such as hydrochloric and acetic acids to be significantly less inhibitory. A very interesting study involving screening 17 strains of lactobacilli against 10 strains of *H. pylori* revealed that the inhibition was related to acid production and lowering of the pH. A specific strain, *L. acidophilus* CRL 639, showed autolytic behaviour and the bactericidal effect was attributed to a proteinaceous substance released by the lysed cells (Lorca *et al*., 2001). *L. salivarius* WB 1004

inhibited the attachment of *H. pylori* to murine and gastric epithelial cells and reduced interleukin-8 (IL-8) release in vitro. Interestingly, the ingestion of the probiotics helped eliminate colonization by *H. pylori* in the infected murine model (Kabir *et al*., 1997).

Some probiotic, non-lactic strains such as *Weissella confusa* produce a class II, nonproteinaceous material which is active against *H. pylori* (Nam *et al*., 2002). Similarly, *Bacillus subtilis* produces antibiotics, identified as amicoumacin A and a nonamicoumacin antibiotic against *H. pylori* in vitro (Pinchuk *et al*., 2001). Probiotics also compete for adhesive or binding sites on the gastric membrane, thus preventing mucosal adhesion by *Helicobacter*. *L. reuteri, W. confusa, L. casei rhamnosus* and many other bacteria have competitively inhibited binding of *H. pylori* thus displaying antimicrobial behaviour. *Bacillus clausii* was administered to symptom-free *H. pylori* positive patients, in a randomized, double-blind, placebo controlled trial. *B. clausii* reduced the common side-effects associated with anti- *H. pylori* antibiotic therapy (Nista *et al*., 2004). Presented in Table 1.21 is a listing of strains of probiotic bacteria used in treatment of *Helicobacter pylori* infection.

Table 1.21 Probiotic cultures used to treat *Helicobacter pylori* infection

Strains	Author and Year
***L. acidophilus* (*johnsonii*) La1**	Michetti *et al*. (1999)
L. acidophilus	Canducci *et al*. (2001)
***L. acidophilus* CRL 639**	Lorca *et al*. (2001)
L. casei* strain *Shirota	Cats *et al*. (2003)
L. casei* strain *Shirota* and *L. acidophilus	Tursi *et al*. (2004)
	Sgouras *et al*. (2004)
***L. casei* subsp. DG**	Sakamoto *et al*.(2001)
***L. gasseri* OLL2716**	Armuzzi *et al*. (2001)
L. GG	Linsalata *et al*. (2004)
L. brevis	
***L. rhamnosus* (R0011) and *L. acidophilus* (R0052)**	Johnson-Henry *et al*. (2004)
	Kabir *et al*. (1997)
***L. salivarius* WB1004**	Aiba *et al*. (1998)
L. salivarius	Mukai *et al*. (2002)
L. reuteri	Pena *et al*. (2005)
L. reuteri* and *L. paracasei	
Lactobacilli and Bifidobacteria containing yoghurt	Sheu *et al*. (2002), Wang *et al*. (2004)
	Nam *et al*. (2002)
Weisella confusa	Pinchuk *et al*. (2001)
Bacillus subtilis	Nista *et al*. (2004)
Bacillus clausii	

2.0. DISCRIMINATION OF DAIRY ISOLATES OF THE *LACTOBACILLUS CASEI* GROUP

2.1. INTRODUCTION

Strains of *Lactobacillus* are found in many environments. Four closely related facultatively heterofermentative species (*Lactobacillus casei*, *L. paracasei*, *L. rhamnosus* and *L. zeae*) form a taxonomic group and are historically difficult to distinguish from each other using traditional methods. Many strains have very similar physiological properties and nutritional requirements, and grow under similar environmental conditions. The *Lactobacillus casei* related group contains a number of well known probiotic strains including *L. casei* Shirota (Sugita and Togawa, 1994) and *L. rhamnosus* GG (Saxelin, 1997). The identification of *Lactobacillus* strains within this group (*Lactobacillus casei, L. paracasei, L. rhamnosus* and *L. zeae*) is important for both basic studies and application in food industries. Traditional phonotypical tests for *Lactobacillus* identification can be difficult to interpret. The techniques are also time consuming and results are often ambiguous (Charteris *et al*., 1997; Hammes *et al.,* 1992). While this group of lactobacilli can be readily distinguished from other members of the *Lactobacillus* genus by fermentation profiles (Hammes *et al*., 1992), it is not possible to unequivocally distinguish between these four species on the basis of fermentation patterns. They form a closely related taxonomic group. In recent years, the taxonomy of this group has changed considerably with increasing knowledge of genomic structure and phylogenetic relations between *Lactobacillus* species(Klein *et al*., 1998; Tilsala-Timsajarvi *et al*., 1999).

Strains from this group belong to the normal intestinal flora and potentially have

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beneficial probiotic effects on human and animal health (Ouwehand et al., 2002), but some strains have been observed to be involved in opportunistic infections (Adams, 1999). Some bacteria of this group are major components of starter cultures for cheeses and yoghurts, and are also found naturally in raw milk and in high numbers in matured cheeses, where they contribute to positive and negative aspects of cheese maturation and flavor development (Khalid and Marth, 1990; Fitzsimons *et al*., 1999).

The classification scheme applied to these bacteria has changed considerably in recent decades. The changes are largely based on improved knowledge of gene sequences, and are intended to make the classification scheme accurately reflect natural bacterial groups and the evolutionary relationships between them. Such changes are entirely sensible to taxonomists and evolutionary biologists but can complicate interpretation of the literature and cause confusion for industrial users of these bacteria and for those marketing or consuming products containing them.

Several DNA homology studies (*e.g.* Collins et *al*., 1989) resulted in 3 species being recognized (*L. casei*, *L. paracasei* and *L. rhamnosus*) instead of a single '*L. casei*' taxon. Within a few years, further studies resulted in proposal of a new species, *L. zeae* (Dicks et al., 1996), and to the suggestion (Dicks et al., 1996; Dellaglio *et al*., 2002) that some strains currently classified as *L. casei* and *L. zeae* should be regarded as one and the same species (and given the name *L. zeae*) and that many strains currently known as *L. casei* and *L. paracasei* should be reclassified into a single species (and given the name *L. casei*, with the name *L. paracasei* abandoned). The Judicial Commission of the International Committee on Systematics of Prokaryotes has yet to publish an opinion on this proposal (De Vos *et al*., 2005). The recent literature must be interpreted carefully, as some authors have adopted this new classification (*e.g.* Dellaglio *et al*., 2002; Dobson *et al*., 2004) but others have not (*e.g.*

Mori et al., 1997; Ward et al., 1999; Vásquez et al., 2005; this study). Commercial strain descriptions often ignore the recommendations of taxonomists, and the classification '*L. casei*' is sometimes loosely applied to strains of any of these species.

There is a need for rapid and reliable species-specific identification including PCR methods, 16S rRNA gene sequencing and restriction enzyme analysis (e.g., pulsed –field gel electrophoresis (PFGE)) (Schwartz and Cantor, 1984; Tanskanen *et al*., 1990). In industrial application of lactobacilli, it is important to have tools with which to identify particular strains, to understand the relationships between strains, to monitor the genetic stability of strains, and to classify them into recognizable species with due reference to the current taxonomy of the organisms. As part of broader work on the potential probiotic properties of lactobacilli (Desai *et al*., 2004), the aims of this study were to examine the effectiveness of carbohydrate fermentation tests, species-specific polymerase chain reaction (PCR) and pulsed-field gel electrophoresis (PFGE) of genomic restriction fragments for discriminating food industry strains of the *L. casei* group.

2.2 MATERIALS AND METHODS

2.2.1 Bacterial Strains

Bacterial strains used in this study (Table 1) were cultured using MRS broth (static cultures) or MRS agar (Oxoid, Basingstoke, UK) at 37°C. All strains grew well without the use of anaerobic conditions. Type strains (*i.e.* recognized reference strains) were included in the study. Species epithets are used in this report in accordance with the four-species scheme (*L. casei*, *L. zeae, L. paracasei*, *L. rhamnosus*), with cross-reference to the alternative three-species scheme (*L. casei, L. zeae*, *L. rhamnosus*) where appropriate.

2.2.2 Species-specific PCR

PCR primers designed on the basis of ribosomal RNA operon sequences are described in Table 2. Oligonucleotide primers used for PCR were synthesized by Life Technologies (Melbourne, VIC, Australia). PCR reactions (50 μL, using reagents from Roche Diagnostics, Castle Hill, NSW, Australia) were performed in 10 m*M* Tris-HCl (pH 8.3), 1.5 m*M* $MgCl_2$, 50 m*M* KCl in the presence of *Taq* DNA polymerase (1 U), deoxynucleotide triphosphates (200 μ*M* each), two primers (0.5 μ*M* each) and 1 μL of bacterial genomic DNA prepared (by the method of Pu et al., 2002) from a fresh colony grown on the surface of MRS agar. A PTC thermocycler (M J Research, MA, USA) was used. Initial denaturation was carried out at 94ºC for 3 min, followed by 30 cycles of 45 s at 50°C, 60 s at 72°C and 45 s at 94°C, with a final product extension for 5 min at 72ºC. The PCR products were analysed by 1% (wt/vol) agarose gel electrophoresis in 0.5×Tris-Borate-EDTA buffer with ethidium bromide (0.5 μg/mL).

2.2.3 *Partial 16S rRNA Gene Sequencing*

PCR products amplified using primers Y1 and Y2 were purified using Wizard columns (Promega, Annandale, NSW, Australia) and their sequences determined with ABI PRISM BigDye Terminator (Applied Biosystems, Scoresby, VIC, Australia) fluorescent

dye-terminator chemistry by the Micromon nucleotide sequencing facility (Monash University, Clayton, VIC, Australia).

2.2.4 Pulsed-Field Gel Electrophoresis (PFGE)

2.2.4.1 Preparation of genomic DNA in agarose blocks

Bacterial genomic DNA was prepared from 1 mL of MRS broth overnight culture, using a modification of the method of Tanskanen *et al*., (1990). Cells were centrifugally harvested, washed and then suspended in 150 µL of NaCl/EDTA/Tris solution (1 *M* NaCl, 10 m*M* EDTA, 10 m*M* Tris-HCl, pH 8.0) at 45ºC. The cell suspension was mixed with 200 µL of low-melting-temperature agarose (2.5% wt/vol; Bio-Rad Laboratories, Regents Park, NSW, Australia) at 45ºC, and then dispensed into molds (Amersham Pharmacia Biotech, Castle Hill, NSW, Australia) and allowed to solidify. Cells immobilized in agarose blocks were incubated overnight at 37ºC in lysis buffer containing 100 m*M* EDTA, 1 *M* NaCl, 10 m*M* Tris-HCl (pH 8.0), 1% lauroyl-sarcosine, 10 mg/mL lysozyme (Sigma, St. Louis, MO, USA) and 30 U/mL mutanolysin (Sigma). Treatment with proteinase K (5 mg/mL; ICN Biochemicals, Seven Hills, NSW, Australia) was performed for 4 days at 42ºC in 100 m*M* EDTA pH 8.0, 1% lauroyl-sarcosine.

2.2.4.2 Restriction enzyme digestion and PFGE.

A 1 mm slice was cut from each agarose block using a sterile razor blade. Slices were equilibrated with 1 m*M* EDTA, 10 m*M* Tris-HCl (pH 8.0) for 1 h, then with two changes (45 min each) of *Not*I restriction enzyme buffer (Roche). Restriction enzyme digestion was carried out with 5U of *Not*I at 37ºC for 18-20 h.

Electrophoresis was carried out with an LKB Pulsaphor CHEF apparatus (Amersham Pharmacia Biotech) in 1.3% agarose with 0.5×Tris-Borate-EDTA buffer at 14ºC and at 290V. Field pulse times were ramped from 1 s to 10 s over 6 h, then 10 s to 20 s over 4 h. The agarose gel was stained with ethidium bromide (0.5 µg/mL) in 0.5×Tris-Borate-EDTA buffer

for 1 h. DNA bands were visualized using 302 nm UV transillumination and photographed with a MultiDoc-It digital imaging system (UVP, Cambridge, U.K.). Electrophoretic band patterns were compared by Jaccard-UPGMA analysis using GelCompar II software (Applied Maths, Sint-Martens-Latem, Belgium) with band-matching optimization and tolerance settings of 1%.

2.2.5 Carbohydrate fermentation tests

API 50CHL test plates and APILABplus software with identification database version 4.0 (Bio-Merieux, Marcy-l'Etoile, France) were used to classify strains biochemically. Further Jaccard-UPGMA analysis was performed on tabulated data using FreeTree 0.9.1.50 (Pavlicek et al., 1999) and TreeView 1.6.6 (Page, 1996) software.

2.3. RESULTS AND DISCUSSION

2.3.1 Use of PCR to distinguish species

PCR primers (Table 2.2) targeting the 16S ribosomal RNA gene and the spacer region between the 16S and 23S rRNA genes were used in this study. Some of these primers had previously been used in limited studies that did not include a full set of species type strains and in which the results of PCR were not corroborated by sequence analysis of the bacterial strains used.

In the present study, a gene region which included the sequences targeted by primers W1, W2, W3 and D1 was PCR amplified from each of the type strains using the Y1-Y2 primer pair, and the DNA sequence from each strain was determined. Sequences were consistent with relevant GenBank sequence entries AY196978 (ATCC393), D86517 (ATCC334), D16552 (ATCC7469) and D86516 (ATCC15820).

PCR was carried out using template DNA from each of the type strains (Table 1) with primer pairs W1-Y2, W2-Y2, W3-Y2, and D1-Y2. In these experiments, the common primer Y2 was used with different specific primers designed on the basis of sequence 'signatures' from the V1 region of the 16S rRNA gene (Mori et al., 1997; Ward and Timmins, 1999; GenBank D86516). A PCR product of approximately 295 bp was amplified from each type strain, depending on which specific primer was used (Table 2.3). It was therefore shown that these primer pairs could distinguish the four bacterial type strains. PCR gel results are shown in (Figure 2.1).

The same PCR assays were applied to 17 industrial isolates from various sources (Table 2.1). Most of these strains had been isolated and phenotypically classified by food industry laboratories between the years 1975 and 1998, and ASCC277 is derived from a strain acquired by the NCIMB in 1958. Ten of these strains had historically been classified as '*L. casei*', a classification which could at the time have included strains which now would be

classified as *L. casei* (*L. zeae* under recent proposals), *L. paracasei* (*L. casei* under recent proposals), *L. zeae* or *L. rhamnosus*.

Using the PCR assay, 13 of the 17 strains were classified as *L. rhamnosus,* including 9 of the 10 strains historically described as *L. casei*. Confirmation of the classification of these strains was achieved by testing all strains in PCR with primer pair Rha(F)-Rha(R) (Table 2.2). This primer pair and the W3-Y2 pair gave the same classification of *L. rhamnosus* strains (results not shown).

Four strains gave positive PCR with the W2-Y2 primer pair, resulting in classification as *L. paracasei* (*L. casei* under recent proposals). None of the industry isolates gave positive PCR with primer pairs W1-Y2 or D1-Y2. This is consistent with molecular taxonomic studies that have defined few strains of *L. casei or L. zeae*.

2.3.2 Partial 16S rRNA gene sequencing

Polymerase chain reaction products amplified using primers Y1 and Y2 were purified and their sequences determined. Available sequences were pruned and blasted (compared) against type strains of type strains sequences of ASCC 290, ASCC 292, ASCC 1521 and ATCC 15820 with genbank entries. DNA sequence from each strain was determined. Sequences were consistent with relevant GenBank sequence entries AY196978 (ATCC393), D86517 (ATCC334), D16552 (ATCC7469) and D86516 (ATCC15820). They were checked for the strains authenticity and purity. They were also aligned and to each other to verify their variable region within each strain shown in (Figure 2.2).

2.3.3 Pulsed-field Gel Electrophoresis

Pulsed-field gel electrophoresis of genomic *Not*I restriction fragments was carried out to discriminate strains at the genetic level. Tynkkynen et al. (1999) used this method and concluded that it was a powerful technique for discriminating *L. rhamnosus* strains. In their study involving human isolates of *L. rhamnosus* they found several isolates to be

indistinguishable from probiotic strain *L. rhamnosus* GG (Saxelin, 1997), perhaps indicating widespread consumption of this commercially-produced strain.

In the present study, inspection revealed 18 different *Not* I restriction fragment patterns from the 21 independent strains studied (Figure 2.3), showing that most of the strains were genuinely different from each other and distinguishable by this method. This complements the PCR methods used, which distinguished *L. paracasei* (*L. casei* under recent proposals) and *L. rhamnosus* strains at the species level.

Strains ASCC290 and CSCC5203 (both representing the type strain of *L. casei* but obtained from independent sources) showed identical fragment patterns, consistent with their recorded identity. Strains ASCC1523 and CSCC2627 also gave indistinguishable patterns, but no common origin for these strains is known. The patterns were also indistinguishable for strains ASCC277, ASCC1181 and ASCC1520. One of these (ASCC277 = NCIMB8963) has long been available from a commercial culture collection.

Small-fragment restriction enzyme pattern comparison has been shown to give useful species distinction of *L. paracasei* from *L. rhamnosus*, and the *L. zeae* type strain from other species, but the *L. casei* type strain could not be distinguished from *L. rhamnosus* (Vásquez et al., 2005). In the present study, using large-fragment PFGE (with an enzyme chosen because it shows differences between strains) the species could not be separated (data not shown). However, after using PCR to make a species assignment, pattern similarities (close strain relationships) within *L. rhamnosus* were readily identified (Fig. 1B) and distinction of *L. paracasei* strains and the type strains of *L. casei* and *L. zeae* could be achieved (Fig.1A). The similarity index (Fig. 1) indicates that *L. casei* and *L. zeae* are perhaps no more distant from each other than are some strains within *L. paracasei* or *L. rhamnosus*.

2.3.4 Biochemical classification

Biochemical identification results from API 50CHL testing are shown in Table 2.1. *L. paracasei* (*L. casei* under recent proposals) and *L. rhamnosus* classifications were consistent with classifications from the PCR assays with one clear exception (ASCC279). The internal quality assessment feature of the API software judged 2 classifications (ASCC526 and CSCC2603) as "unacceptable", implying that the pattern analysis software was unable to make a clear classification. Neither *L. casei* nor *L. zeae* is classifiable using the API proprietary database and software, presumably because few strains have been available for study. The *L. casei* and *L. zeae* type strain fermentation patterns differed from typical *L. paracasei* fermentation patterns by 6 to 9 sugars fermented or not fermented and by 5 sugars from each other. Even so, Jaccard-UPGMA analysis of the fermentation test results was also unable to separate these strains from the other species or from each other (not shown). Such observations illustrate the limitations of biochemical profiling when applied to closely related bacteria with intra-species heterogeneity.

2.3.5 Taxonomic considerations

The phylogenetic relationships between the various bacteria of this group are generally not in dispute, but there is debate over where species boundaries should be drawn and what names should be attached to those species. The results of this study are consistent with the prevailing view that (i) *L. rhamnosus* is a common and readily distinguishable species, (ii) a second common and readily distinguishable species exists, herein referred to as *L. paracasei*, but proposed by some (Dellaglio *et al*., 2002) to be known as *L. casei*, and (iii) relatively uncommon types also exist. These *L. casei/L. zeae* types are undoubtedly closely related to each other (Mori *et al*., 1997; Dobson *et al*., 2004; Vásquez *et al*., 2005), plausibly justifying reclassification of these bacteria into a single species (Dellaglio *et al*., 2002).

At the heart of the nomenclature debate is the proposal to reclassify the type strain of *L. casei* (ATCC393 and equivalents, in this study ASCC290 and CSCC5203) as *L. zeae*. Species are conventionally defined largely in terms of their type strains, and so this reclassification would be a highly unusual step. The phylogenetic relationships could equally be accommodated by merging *L. casei* and *L. zeae* under the name *L. casei* (with ATCC393 as the type strain) and reclassifying some strains currently held in collections as *L. casei* to *L. paracasei.* This would not involve the controversial act of removing a type strain from its species.

2.3.6 Practical species and strain discrimination

Mori et al. (1997) proposed that the sequences of 16S ribosomal RNA genes could distinguish species within the *L. casei* group, and established 'signature' sequence features that could be used as PCR targets for species-specific product amplification (*e.g.* Ward and Timmins, 1999). This approach has been extended in the present work to permit four groups to be discriminated. PCR conditions (particularly the 'anneal' temperature; data not shown) are crucial to achieving clear discrimination, and might require adjustment for use with different PCR reaction mixes or thermal cycling devices. The use of four separate PCR reactions (plus a negative control to which no template DNA is added) provides a set of positive/negative test results that are easily interpreted and provide immediate confirmation of PCR specificity or of any 'cross-reaction'. The methods used in this study (PCR followed by electrophoresis) are now available to many food laboratories, and the ease of interpretation is a considerable advantage over conventional tests such as fermentation pattern analysis. The species-specific primers could also form the basis of a 'probe oligonucleotide' set for use in a single-tube quantitative PCR assay using fluorescence-detection real-time PCR. The positive/negative aspect of the test would also be a useful confirmation of specificity in a real-time assay system.

The combination of four-reaction PCR and PFGE is a workable approach for strain classification and identification, but the results must be interpreted carefully. The results obtained in this study are consistent with the conclusion of Mori et al. (1997), Ward and Timmins (1999) and Vásquez et al. (2001), that *L. paracasei* and *L. rhamnosus* could readily be identified and that *L. casei* could be distinguished from *L. zeae* on the basis of aspects of their 16S rRNA gene sequences. Both are readily distinguished from *L. paracasei* and *L. rhamnosus.* However, Vásquez et al. (2005) reported a more detailed sequence analysis showing that the multiple copies the 16S rRNA gene within a single strain are not always identical. Notably, intra-strain sequence heterogeneity was observed within the type strains of *L. casei*, *L. paracasei* and *L. zeae*. Inter-strain sequence polymorphism was observed within these species and within *L. rhamnosus*, further complicating interpretation of available sequence data and identification of unambiguous signature sequences. Some of this heterogeneity is within the region targeted by the primers W1, W2, W3 and D1 (at positions 96, 97, 99, 105, and 110 as described by Vásquez et al., 2005). The results of the present study suggest this heterogeneity does not interfere with this PCR-based classification of strains of *L. paracasei* and *L. rhamnosus*, nor does it interfere with distinction between the type strains of *L. casei* and *L. zeae.* The full extent of inter- and intra-strain heterogeneity within the latter species (whether they are regarded as two species or merged into a single species) is not known because so few strains have been studied. It is possible that the primers W1 and D1 target only two of many possible sequence variants. Evaluation with other strains would be required to be sure that no problems existed, but the multiple positive/negative aspect of the test scheme would alert the experimenter to any cross-reactions that might occur.

2.3.7 Reclassification of archive isolates

Strains described in the historical literature or held in archival collections require re-examination and possible reclassification before they can be properly described and compared with recent isolates and the recent literature. In this study, 9 strains historically described as *L. casei* were reclassified as *L. rhamnosus.*

Ward and Timmins (1999) examined '*L. casei* group' isolates from cheeses, and reported *L. rhamnosus* and a preponderance of *L. paracasei*, but no *L. casei.* The isolates tested in the present study came from cheeses but also from a range of non-cheese sources, with *L. rhamnosus* as the most common species. Considered together, these studies indicate that *L. casei* (as currently defined) is not common in dairy environments or in dairy manufacturing. *L. paracasei* and *L. rhamnosus* are the common cheese isolates from the *L. casei* group and, in this study, *L. rhamnosus* was identified from cultures and other dairy sources.

2.4 Tables

Table 2.1. Bacterial strains used in this study

Strains	Source, equivalent designations, notes[1]	Classification[2]	
		API[3]	PCR[4]
ASCC 290	NCDO161 = NCIMB11970 = ATCC393 from FRI (1993); *L. casei* type strain	-	C
CSCC 5203	LMG6904 = ATCC393, from LMG (1996); *L. casei* type strain	-	C
ATCC 15820	From ATCC (2001); *L. zeae* type strain	-	Z
ASCC 292	NCDO151 = NCIMB700151 = ATCC25302 from FRI (1993); *L. paracasei* type strain	P	P
ASCC 1521	Single-colony isolate from CSCC2625 = ATCC7469 (1992); *L. rhamnosus* type strain	R	R
Other isolates			
ASCC 295	From FRI (1993); isolated from Australian mature Cheddar cheese as *L. casei* subsp. *pseudoplantarum* (subspecies later reclassified as *L. paracasei)*	P	P
ASCC 526	Isolated from a commercial fermented milk drink (1994) as *L. paracasei*	-	P
ASCC 1180	Isolated from Canadian mature Cheddar cheese (1998) as *L. paracasei*	P	P
ASCC 279	From FRI (1993); isolated from Australian mature Cheddar cheese as *L. casei*	R	P
ASCC192	Canadian isolate, received as *L. rhamnosus* (1992)	R	R
ASCC 277	From FRI (1993) = NCIMB8963 (acquired 1958), received as *L. casei*	R	R
ASCC 1002	Isolated from English Farmhouse Cheddar cheese (1996) as *L. rhamnosus*	R	R
ASCC 1181	Isolated from English Double Gloucester cheese (1998) as *L. rhamnosus*	R	R
ASCC 1519	From CSIRO (2000), isolated (1979) from bovine rennet as *L. casei*	R	R
ASCC 1520	From CSIRO (2000), isolated (1982) from Australian mozzarella cheese as *L. casei*	R	R
ASCC 1522	From CSIRO (2000), isolated (1995) from a commercial probiotic preparation as *L. casei*	R	R
ASCC 1523	From CSIRO (2000), isolated (1996) from a European fermented milk drink as *L. casei*	R	R
CSCC 2603	From NZDRI (1975) via CSIRO, isolated from bovine rennet as *L. casei*	-	R
CSCC 2605	From NZDRI (1975) via CSIRO, received as *L. rhamnosus*	R	R
CSCC 2607	Isolated from Australian mozzarella cheese (1984) as *L. casei*	R	R
CSCC 2627	Isolated from a commercial probiotic preparation (1992) as *L. casei*	R	R
CSCC 5376	Isolated from a commercial freeze-dried culture (1997) as *L. casei*	R	R

Table 2.1.

[1] Abbreviations:

ASCC = Australian Starter Culture Collection, Australian Starter Culture Research Centre; ATCC = American Type Culture Collection; CSIRO = Commonwealth Scientific and Industrial Research Organisation, Australia; CSCC = CSIRO Starter Culture Collection; FRI = Food Research Institute, Australia; LMG = Belgian Co-ordinated Collections of Micro-organisms, Laboratorium Mikrobiologie, Gent; NCDO = National Collection of Dairy Organisms, UK (available through NCIMB); NCIMB = National Collection of Industrial, Marine and Food Bacteria, UK; NZDRI = New Zealand Dairy Research Institute.

[2] *Lactobacillus* species classification: C = *L. casei*, P = *L. paracasei*, R = *L. rhamnosus*, Z = *L. zeae.*

[3] Classification based on API 50CHL test plates. Dashes indicate no classification.

[4] Classification based on PCR with primer sets 1 and 2 (Table 2.2).

Table 2.2. 16S ribosomal RNA gene PCR primers used in this study

Name	Sequence 5'-3'	Comments, references
Primer set 1		
Y1	TGGCTCAGAACGAACGCTAGGCCCG	'Universal' primer, targets a widely-conserved sequence for amplification from bacterial 16S rRNA gene (Young et al. (1991).
Y2	CCCACTGCTGCCTCCCGTAGGAGT	'Universal' primer, targets a widely-conserved sequence for amplification from bacterial 16S rRNA gene (Young et al. (1991).
W1	TGCACTGAGATTCGACTTAA	Targets 16S rRNA gene; 'casei' primer of Ward et al. (1999).
W2	CACCGAGATTCAACATGG	Targets 16S rRNA gene; 'para' primer of Ward et al. (1999).
W3	TGCATCTTGATTTAATTTTG	Targets 16S rRNA gene; 'rham' primer of Ward et al. (1999).
D1	TGCATCGTGATTCAACTTAA	Targets 16S rRNA gene (this study); analogous to W1 but designed on basis of sequence from ATCC15820 (GenBank D86516).
Primer set 2		
Rha(F)	CTTGCATCTTGATTTAATTTTG	Targets *L. rhamnosus* 16S rRNA gene (Tynkkenen et al., 1999). Targets same region as the W3 primer.
Rha(R)	CCGTCAATTCCTTTGAGTTT	Targets *L. rhamnosus* 16S rRNA gene (Tynkkenen et al., 1999).

Table 2.3. PCR analysis of *Lactobacillus* type strains

Primers used	Type strains			
	L. casei ASCC290	*L. paracasei* ASCC292	*L.rhamnosus* ASCC1521	*L. zeae* ATCC15820
W1-Y2	+	-	-	-
W2-Y2	-	+	-	-
W3-Y2	-	-	+	-
D1-Y2	-	-	-	+

2.5 Figures

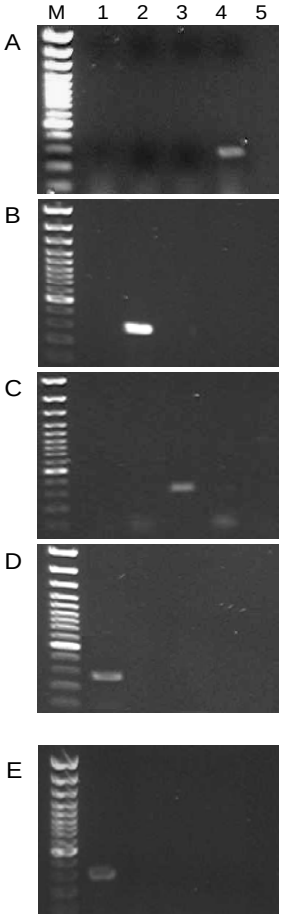

Figure 2.1 PCR gel figures shows positive results: Gel A is for Positive with *L. zeae* (ATCC 15820), Gel B is positive with *L. paracasei* (ASCC 292), Gel C is positive with *L. rhamnosus* (ASCC 1521) and Gel C and D are positive with *L. casei* (ASCC290 and CSCC 5203) respectively.

```
Sequence      1          11         21         31         41         51
1             TGGTCGATGA ACGGTGCTTG CACTGAGATT CAACTTAAAA CGAGTGGCGG ACGGGTGAGT
2             TGGTCGATGA ACGGTGCTTG CATTGTGATT CAACTTAAAA CGAGTGGCGG ACGGGTGAGT
3             TCGTTGATGA TCGGTGCTTG CACCGAGATT CAACATGGAA CGAGTGGCGG ACGGGTGAGT
4             TGATTATTGA AAGGTGCTTG CATCTTGATT TAATTTTGAA CGAGTGGCGG ACGGGTGAGT

 Sequence    61         71         81         91         101        111
1             AACACGTGGG TAACCTGCCC TTAAGTGGGG GATAACATTT GGAAACAGAT GCTAATACCG
2             AACACGTGGG TAACCTGCCC TTAAGTGGGG GATAACATTT GGAAACAGAT GCTAATACCG
3             AACACGTGGG TAACCTGCCC TTAAGTGGGG GATAACATTT GGAAACAGAT GCTAATACCG
4             AACACGTGGG TAACCTGCCC TTAAGTGGGG GATAACATTT GGAAACAGAT GCTAATACCG

 Sequence    121        131        141        151        161        171
1             CATAAATCCA AGAACCGCAT GGTTCTTGGC TGAAAGATGG CGCAAGCTAT CGCTTTTGGA
2             CATAAATCCA AGAACCGCAT GGTTCTTGGC TGAAAGATGG CGTAAGCTAT CGCTTTTGGA
3             CATAGATCCA AGAACCGCAT GGTTCTTGGC TGAAAGATGG CGTAAGCTAT CGCTTTTGGA
4             CATAAATCCA AGAACCGCAT GGTTCTTGGC TGAAAGATGG CGTAAGCTAT CGCTTTTGGA

 Sequence    181        191        201        211        221        231
1             TGGACCCGCG GCGTATTAGC TAGTTGGTGA GGTAACGGCT CACCAAGGCG ATGATACGTA
2             TGGACCCGCG GCGTATTAGC TAGTTGGTGA GGTAACGGCT CACCAAGGCG ATGATACGTA
3             TGGACCCGCG GCGTATTAGC TAGTTGGTGA GGTAACGGCT CACCAAGGCG ATGATACGTA
4             TGGACCCGCG GCGTATTAGC TAGTTGGTGA GGTAACGGCT CACCAAGGCA ATGATACGTA
```

Sequence	241	251	261	271	281	291
1	GCCGAACTGA	GAGGTTGATC	GGCCACATTG	GGACTGAGAC	ACGGCCCAAA	CTCCTACGGG
2	GCCGAACTGA	GAGGTTGATC	GGCCACATTG	GGACTGAGAC	ACGGCCCAAA	CTCCTACGGG
3	GCCGAACTGA	GAGGTTGATC	GGCCACATTG	GGACTGAGAC	ACGGCCCAAA	CTCCTACGGG
4	GCCGAACTGA	GAGGTTGATC	GGCCACATTG	GGACTGAGAC	ACGGCCCAAA	CTCCTACGGG

Sequence	301
1	AGG
2	AGG
3	AGG
4	AGG

Figure 2.2. Four type strains sequences derived by 16S rRNA gene sequencing .1) is *L. casei*, 2) *L. zeae*, 3) *L. paracasei*, 4) *L. rhamnosus*. They have been aligned to each other. Variable region has been underlined to show difference within each strain.

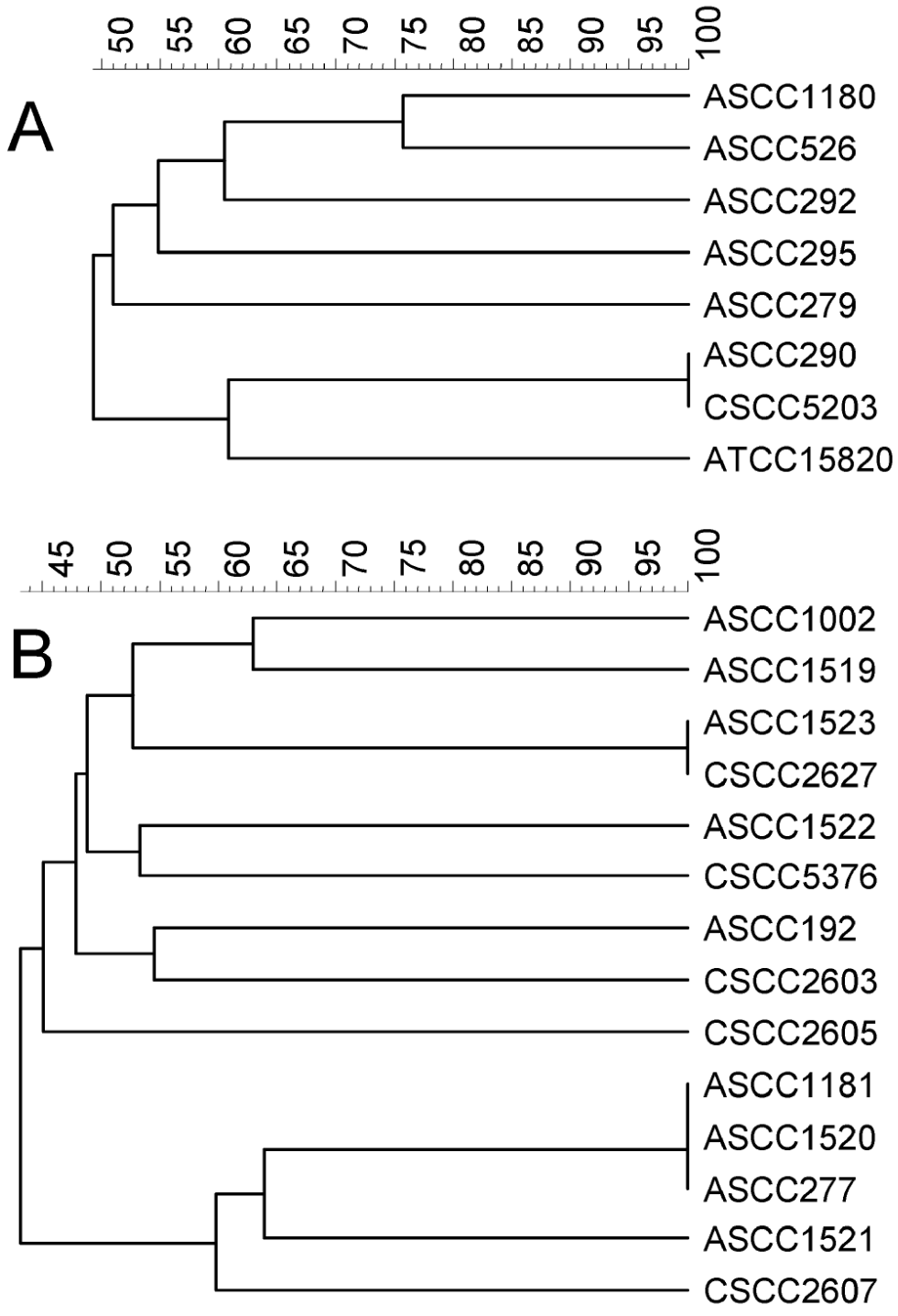
A
50
55
60
65
70
75
80
85
90
95
100
ASCC1180
ASCC526
ASCC292
ASCC295
ASCC279
ASCC290
CSCC5203
ATCC15820
B
45
50
55
60
65
70
75
80
85
90
95
100
ASCC1002
ASCC1519
ASCC1523
CSCC2627
ASCC1522
CSCC5376
ASCC192
CSCC2603
CSCC2605
ASCC1181
ASCC1520
ASCC277
ASCC1521
CSCC2607

Figure 2.2. Dendrograms derived from UPGMA analysis of Jaccard similarities in *Not*I genomic fragment patterns obtained in PFGE. The similarity scale (%) is shown. Panel A: *L. paracasei*, *L. casei* and *L. zeae*; Panel B: *L. rhamnosus*. ASCC= Australian Starter Culture Collection, Australian Starter culture Reasearch Centre (Werribee, Victoria, Australia); CSCC= Commonwealth Scientific and Industrial Research Organisation (CSIRO) Starter Culture Collection (Highett, Victoria, Australia); ATCC= American Type Culture Collection (Manassas, VA)

2.6 CONCLUSIONS

In this study, we have confirmed that a set of simple PCR tests based on 16S rRNA gene sequences, coupled with PFGE, can be used to distinguish *Lactobacillus* strains of the *L. casei* group. The usefulness of sugar fermentation tests is limited to identification of *L. paracasei* and *L. rhamnosus*. The results indicate that *L. casei* and *L. zeae* are not common in dairy environments and that (at least within the collection studied) the prevalence of *L. rhamnosus* has been historically underestimated. Reclassification of strains described in the literature and in industry practice is recommended.

3.0 INVESTIGATION OF TOLERANCE OF *LACTOBACILLUS* STRAINS TO THE PRESENCE OF ACID, BILE SALTS AND DECONJUGATED BILE SALTS

3.1 Introduction

Traditionally probiotic bacteria have been utilized in dairy products such as milk or yoghurt and it has been hypothesized that milk enhances probiotic efficacy by providing lactose as a substrate (Succi *et al.*, 2005). At present, a large number of dairy products present on market and are being promoted with health claims based on several characteristics of selected strains of lactic acid bacteria, particularly belonging to the genera *Lactobacillus* and *Bifidobacterium* (Shah, 2000). Probiotic bacteria constitute a major part of the natural micro flora of human intestine and when present in sufficient numbers create a healthy equilibrium between beneficial and potentially harmful microflora in the gut (Gilland *et al*., 1977; Beck *et al.*, 1961). To provide health benefits, the suggested concentration for probiotic bacteria is 10^6 CFU/g of a product (Lankaputra *et al.*, 1995).

Viability and survival of probiotic bacteria are the most important parameters in order to provide therapeutic functions. A number of factors have been claimed to affect the viability of probiotic bacteria in dairy foods such as yoghurt and fermented milks, including low pH and refrigerated storage (Shah, 2000). Micro-organisms ingested with food begin their journey to the lower intestinal tract via the mouth and are exposed during their transit through the gastrointestinal tract to successive stress factors that influence their survival (Marteau *et al.*, 1993; Simon & Gorbach, 1987). The time from entrance to release from the stomach is

about 90 min, but further digestive processes have longer residence times (Berrada *et al.*, 1991).

Cellular stress begins in the stomach, which has pH as low as 1.5 (Lankaputhra & Shah 1995). Bile secreted in the small intestine reduces the survival of bacteria by destroying their cell membranes, whose major components are lipids and fatty acids and these modifications may affect not only the cell permeability and viability, but also the interactions between the membranes and the environment (Gilliland, 1987; Gilliland *et al.*, 1984). Therefore before a probiotic can benefit human health it must fulfill several criteria such as the ability to tolerate acid and bile salts as well as to grow in the lower intestinal tract (Pereira & Gibson, 2002).

One of the important characteristics of the microorganisms is their ability to survive through the acidic conditions in the human stomach and bile concentrations in the intestine and colonise in the gut. For this to occur, viable cells of *Lactobacillus* must be able to survive the harsh condition of acidity and bile concentration commonly encountered in the gastrointestinal tract of humans.

In recent years, interest has been raised in the possibility of using bile salt deconjugation by lactic acid bacteria to lower serum cholesterol level in hypercholesterolemic patients and prevent hypercholesterolemia in people (De smet *et al.*, 1995). Ahn *et al.* (2003) found precipitated halo and opaque granular white material on agar plugs around *L. acidophilus* colonies, which were confirmed to be cholate, chenodeoxycholate and deoxycholate, produced by the deconjugation of taurocholic acid. The major route of cholesterol excretion from humans and other animals is through feces. Cholesterol is the precursor of primary bile salts in the gall bladder for secretion in the gastrointestinal tract (Corzo & Gilliland, 1999). Conjugated bile salts are secreted into small intestine for absorption of dietary fat, hydrophobic vitamins and other fat-soluble compounds. A small

fraction of bile salts that are not absorbed is lost as free bile salts in feces. Free bile salts were less soluble than conjugates bile salts, resulting in lower absorption in the intestinal lumen (Center, 1993). At the physiological pH of the intestinal lumen, deconjugated bile salts can be transported through the epithelium and into the blood stream of the host, or precipitated (Wong *et al.,* 1994). Thus, in a steady-state situation, deconjugation of bile salts can reduce serum cholesterol levels by increasing the formation of new bile salts that are needed to replace those that have escaped the enterohepatic circulation (Reynier *et al.,* 1981). Experiments with germ-free rats have shown that bile salt deconjugation by *B. longum* increase bile salt excretion (Chikai *et al.,* 1987).

The objectives of this study were to evaluate the tolerance of twenty-two strains of *Lactobacillus* to acidic conditions, bile concentration that commonly exist in the human stomach and the intestine. Seven strains of *Lactobacillus* ASCC 1520, ASCC 1521, ASCC 279, ASCC 290, ASCC 292, CSCC 2607 and ATCC 15820 were also examined for their bile salt deconjugation ability.

3.2 Material and Methods

Twenty-two strains of *Lactobacillus casei* group were obtained from Australian Starter Culture Research Centre (Werribee, Australia). They were cultured using de Man, Rogosa, and Sharpe (MRS) broth (Oxoid, Basingstoke, UK) at 37^0C. All strains grew well without the use of anaerobic conditions. Type strains (i.e., recognized reference strains) were included in the study. Stock cultures were stored in 40% glycerol at -80^0C. All of the organisms were subcultured three times consequently prior to use in sterile MRS broth using 1% inoculum and 20 h incubation at 37^0C.

3.2.1 Survival of Lactobacillus spp *under acidic conditions*

To evaluate the survival of twenty two strains of *Lactobacillus casei* under acidic conditions, aliquots of each active cultures grown in MRS broth for 20 h at 37^0C were adjusted to pH 3.0, 2.5, 2.0 and 1.5 with 5 N HCl and incubated at 37^0C for 3 h. Samples were taken every hour for 3 h and the viable number of *L. casei* were enumerated by pour plate counts of all samples using 10-fold serial dilutions prepared in 0.1% peptone water. The experiment and analysis were carried out in triplicate. The results shown are the averages of at least two replicates.

3.2.2 Survival of Lactobacillus spp *in the presence of bile salts*

To evaluate the survival of twenty two strains of *Lactobacillus casei* in the presence of bile salts, aliquots of active cultures grown in MRS broth for 20 h at 37^0C were adjusted to pH 4.5 with sterile 1 N HCl or 1 N NaOH, depending on the final pH of the culture after 20 h of incubation. Concentrated bile solution was prepared separately by dissolving powdered bile extract (Oxoid). Bile solution was then filter sterilized by 5µ filter and was added to two of the cultures to achieve a final concentration of 1.0% and 1.5% and the third culture served as a control sample. The culture were incubated at 37^0C for 3 h. Samples were taken prior to adding bile solution and then every hour for 3 h. Viable counts of *L. casei* strains were

determined by pour plate counts of all the samples using 10-fold serial dilutions prepared in 0.1% peptone water. The experiment and analysis were carried out in triplicate. The results shown are the averages of at least two replicates.

3.2.3 Enumeration of Lactobacillus spp

de Man Rogosa and Sharpe (MRS) agar (Oxoid) was used for the enumeration of *L. casei*. Cultures were serially diluted from 10^{-1} to 10^{-8} in 0.1% sterile peptone water. One millilitre aliquots of the dilutions were pour plated with MRS agar and incubated aerobically at 37^0C for 72 h. All colonies were counted and recorded as colony forming units (CFU) per gram of culture.

All the experiments and analyses were duplicated. The results shown are the average of at least two replicates.

3.2.4 Deconjugation of sodium glycocholate and sodium taurocholate by *Lactobacilli*

Seven high acid and bile resistant *Lactobacillus* strains were tested for their deconjugation activity. Ten-milliliter volumes of MRS broth were supplemented with 6 mM sodium taurocholate, 6 mM sodium glycocholate or a combination of sodium glycocholate and sodium taurocholate at 2.8 and 1.2 mM, respectively. Individual bile salt were added as 6 mM each, because it resembles the concentrations prevailing in the human small intestine (Brashears *et al*., 1998), while bile mixtures contained 2.8 mM sodium glycocholate and 1.2 mM sodium taurocholate, because they resembles the molar ratio of he two salts and human bile (Sandine, 1979). Each strain was inoculated at the 1% level and incubated aerobically at 37^0C for 20 h. Bile salt deconjugation ability was based on release of deconjugated bile and the modified method of Irwin, Johnson, and Kopalo (1944) was used to measure the amount of free cholic acid released by the organisms. Briefly, 10 mL culture of each organism after the incubation period was adjusted to pH 7.0 with NaOH (1 N). Cells were centrifuged at 10000 *g* (Microspin 24, Sorvall Instruments, Melbourne, Australia) at 4^0C for 10 min.

Supernatant obtained was adjusted to pH 1.0 with HCl (10 N). One milliliter of the supernatant was added with 2 mL of ethyl acetate and the mixture was vortexed for 1 min. Two milliliter of the ethyl acetate layer was transferred into glass tube and evaporated under nitrogen at 60^0C. The residue was immediately dissolved in 1 mL of NaOH (0.01N). After complete mixing, 1 mL of furfuraldehyde (1%) and 1 mL of H_2SO_4 (16N) were added and the mixture was vortexed for 1 min. The sample was then heated in water bath at 65^0C. After cooling 2 mL glacial acetic acid was added and the mixture was vortexed for 1 min. OD was taken at 660 nm (Pharmacia Novascope II, Cambridge, England). Standard cholic acid (Sigma Chemical Co., St Louis, MO, USA) was used to determine the amount of cholic acid released from samples. All experiments were replicated twice.

3.3 Results and Discussion

3.3.1 Survival of Lactobacillus strains under acidic condition

Different regions of the gastrointestinal tract have varying acid levels. Stomach and the regions after stomach have the highest acidity and the pH of these areas may fall to as low as pH 1.5. In order to be used as beneficial adjuncts, *Lactobacillus* must be able to survive these harsh conditions and colonise in the gut.

The survival of twenty two different *Lactobacillus* strains under acidic conditions (pH 1.5, 2.0, 2.5 and 3.0) are illustrated in Table 3.1 to 3.4. In general, the number of survivors of all cultures during 3 h of incubation decreased at all pH conditions. The viable count (log) cfu/g was substantial decreased especially at pH 2.0 and lower. ASCC 292 showed the highest viability followed by ASCC 290, 1520. ATCC 15820, ASCC 2607, 279 and 1521 have also shown moderate activity even at pH 1.5. Fifteen remaining strains have shown no activity after 0 h. As shown in Table 3.2 at pH 2.0, ASCC 1002 has shown lowest viability followed by ASCC 1522 & 295. While 13 remaining *Lactobacillus* strains have shown fair activity up to 2 h of incubation.

Table 3.3 exemplifies viability of *Lactobacillus* strains at pH 2.5. All twenty two strains had shown their viable count (log) cfu/g during 3 h of incubation. All strains decreased their viable count after 3 h of incubation except ASCC 279, 1520 & CSCC 2607. ASCC 279, 1520 & CSCC 2607 shown higher viable count (log) cfu/g at 3 h of incubation.

Table 3.4 shows the viable count of *Lactobacillus* strains at pH 3.0. ASCC 279, 1520 & CSCC 2607 have shown greater viable count (log) cfu/g at 3 h of incubation similar to Table 3.3. Eighteen other *Lactobacillus* strains viable count declined after 3 h of incubation.

3.3.2 Survival of Lactobacillus strains in the presence of bile

Gastrointestinal systems have varying concentrations of bile. The rate of secretion of bile and the concentration of bile in different regions of the intestine vary, depending mainly on the

type of food consumed and it may not be possible to predict the bile concentration in the intestine at any given moment. Davenport (1977) reported that while bile concentrations in the intestine range between 0.5 to 2.0% during first hour of digestion. The levels may decrease during the second hour. Bile concentrations ranging from 0% to 1.5% have been used in several microbiological media for selective isolation of bile tolerant bacteria from mixed cultures.

Tables 3.5 and 3.6 are showing the viable count of twenty two different *Lactobacillus* strains in the bile concentrations of 0.0% and 1.0%. All 22 *Lactobacillus* strains had shown moderate activity at 0% bile concentration up to 3 h of incubation. CSCC 2607 has shown highest growth followed by ATCC 15820 and ASCC 1521 at 1% bile concentration. Remaining *Lactobacillus* strains have shown fair growth up to 3 h of incubation. Results from 1.5% bile concentration are shown in Table 3.7. Only seven *Lactobacillus* strains showed growth up to 3 h of incubation. ASCC 1521 showed highest activity followed by ASCC 290 and ATCC 15820 up to 3 h of incubation. Furthermore ASCC 292, ASCC 279, ASCC 1520 and CSCC 2607 showed reasonable growth during incubation.

3.3.3 Deconjugation of bile salt by Lactobacilli

Bile salt deconjugation activity by *Lactobacillus* strains is shown in Table 8. Bile salt deconjugation was determined by the quantity of cholic acid released, which ranged from 1.16 to 4.70 mM. All seven strains were able to deconjugate both sodium glycocholate and sodium taurocholate at varying levels. Deconjugation activity in broth containing sodium glycocholate was observed. Overall deconjugation was observed to be higher by the strains ASCC 1521 followed by ATCC 15820 and ASCC 292. ASCC 1520 showed lowest deconjugation followed by CSCC 2607. All seven strains showed lower deconjugation of sodium taurocholate compared to sodium glycocholate. ASCC 1521 conjugated highest level followed by ATCC 15820. ASCC 290 shown least capable of deconjugating sodium

taurocholate followed by CSCC 2607. Mixture of sodium glycocholate and sodium taurocholate at similar molar ratio in human bile, all seven strains showed consistent cholic acid secretion. ASCC 1521 also showed highest cholic acid liberation while ASCC 1520 showed lowest deconjugation ability.

This study was carried out only on best seven strains, which were bile resistant in our initial studies. Furthermore there is no data available for bile deconjugation ability on taurine- and glycine- conjugated bile forms by *Lactobacillus* strains. Free bile acids formed by the deconjugation of conjugated bile salts are less soluble and are less likely to be reabsorbed by the intestinal lumen compared to their conjugated equivalent, and are lost from the human body through feces (Center, 1993). Klaver and Van der Meer (1993) theorized that while bile salts were deconjugated and the pH of the fermentation media dropped due to natural acid production by culture, cholesterol micelles destabilized and cholesterol co-precipitated with free bile acids. In this study, all *Lactobacillus* spp. could deconjugate both glycine- and taurine- conjugated bile salts in to cholic acid. However, more glycine –conjugated bile salt deconjugated by all strains than taurine-conjugated bile salt.

3.4 Tables

Table 3.1 Survival of twenty two different *Lactobacillus* strains during three hours at pH 1.5 in HCl solution

	Viable count (Log) cfu / g[1]			
Bacterial strain	0h	1h	2h	3h
ASCC 292	259 ± 7	78 ± 6	0	0
ASCC 290	122± 3	62 ± 8	0	0
ASCC 279	127± 4	35 ± 1	0	0
ASCC1521	126 ± 2	31 ± 1	0	0
ASCC1520	157 ± 5	55 ± 2	0	0
ATCC 15820	191 ± 7	41 ± 5	0	0
CSCC 2607	223 ± 3	37 ± 1	0	0
CSCC5203	150 ± 4	0	0	0
ASCC295	163 ± 6	0	0	0
ASCC 526	189 ± 4	0	0	0
ASCC 1180	190 ± 6	0	0	0
ASCC 277	198 ± 2	0	0	0
ASCC 1002	149 ± 6	0	0	0
ASCC1181	169 ± 4	0	0	0
ASCC 1519	184 ± 3	0	0	0
ASCC 1522	209 ± 2	0	0	0
ASCC 1523	160 ± 8	0	0	0
CSCC2603	224 ± 5	0	0	0
CSCC 2605	252 ± 7	0	0	0
CSCC 2627	173 ± 5	0	0	0
CSCC 5376	183 ± 8	0	0	0
ASCC 192	195 ± 2	0	0	0

± Values refer to standard error of means
Values are means of triplicates from two separate runs, n = 6.
[1] Means viable count (CFU/g).

Table 3.2 Survival of twenty two different *Lactobacillus* strains during three hours at pH 2.0 in HCl solution

	Viable count (Log) cfu / g[1]			
Bacterial strain	0h	1h	2h	3h
ASCC 292	266 ± 7	58 ± 6	55 ± 9	39 ± 3
ASCC 290	166 ± 5	106 ± 1	65 ± 1	35 ± 2
ASCC 279	197 ± 7	109 ± 2	69 ± 2	45 ± 2
ASCC1521	126 ± 4	96 ± 2	84 ± 2	43 ± 1
ASCC1520	160 ± 6	106 ± 4	72 ± 3	43 ± 1
ATCC 15820	213 ± 9	164 ± 4	101 ± 2	64 ± 2
CSCC 2607	238 ± 7	145± 3	96 ± 2	37 ± 3
CSCC5203	163 ± 8	109 ± 2	88 ± 4	0
ASCC295	186 ± 4	155 ± 3	0	0
ASCC 526	204 ± 2	175 ± 6	90 ± 7	0
ASCC 1180	185 ± 4	125 ± 6	86 ± 2	0
ASCC 277	190 ± 4	125 ± 5	52 ± 1	0
ASCC 1002	161 ± 3	0	0	0
ASCC1181	172 ± 4	102 ± 4	85 ± 1	0
ASCC 1519	193 ± 6	116 ± 6	63 ± 2	0
ASCC 1522	201 ± 7	100 ± 4	0	0
ASCC 1523	155 ± 6	93 ± 2	42 ± 1	0
CSCC2603	239 ± 9	160 ± 8	89 ± 4	0
CSCC 2605	227 ± 8	133 ± 4	35 ± 2	0
CSCC 2627	190 ± 2	116 ± 6	52 ± 1	0
CSCC 5376	178 ± 7	112 ± 8	63 ± 3	0
ASCC 192	188 ± 4	125 ± 2	88 ± 2	0

± Values refer to standard error of means
Values are means of triplicates from two separate runs, n = 6.
[1] Means viable count (CFU/g).

Table 3.3 Survival of twenty two different *Lactobacillus* strains during three hours at pH 2.5 in HCl solution

	Viable count (Log) cfu / g[1]			
Bacterial strain	0h	1h	2h	3h
ASCC 292	268 ± 7	208 ± 7	138 ± 3	102 ± 3
ASCC 290	161 ± 2	124 ± 5	108 ± 6	87 ± 2
ASCC 279	164 ± 2	115 ± 2	99 ± 4	104 ± 6
ASCC1521	178 ± 4	138 ± 2	155 ± 4	153 ± 5
ASCC1520	159 ± 7	117 ± 4	91 ± 6	116 ± 1
ATCC 15820	221 ± 6	153 ± 1	144 ± 3	141 ± 2
CSCC 2607	228 ± 4	221 ± 6	223 ± 5	244 ± 9
CSCC5203	195 ± 6	180 ± 4	174 ± 3	50 ± 2
ASCC295	182 ± 4	168 ± 2	161 ± 4	63 ± 2
ASCC 526	163 ± 2	152 ± 3	140 ± 3	30 ± 5
ASCC 1180	181 ± 4	120 ± 1	92 ± 3	80 ± 1
ASCC 277	191 ±3	135 ± 4	103 ± 5	76 ± 2
ASCC 1002	179 ± 7	154 ± 7	99 ± 1	70 ± 2
ASCC1181	178 ± 3	135 ± 4	99 ± 1	80 ± 4
ASCC 1519	169 ±5	101 ± 3	119 ± 2	36 ± 2
ASCC 1522	218 ± 4	152 ± 3	159 ± 4	63 ± 1
ASCC 1523	171 ± 2	63 ± 6	52 ± 4	40 ± 2
CSCC2603	235 ± 7	160 ± 4	152 ± 5	35 ± 2
CSCC 2605	219 ± 5	92 ± 5	79 ± 1	56 ± 3
CSCC 2627	186 ± 6	132 ±3	122 ± 6	41 ± 4
CSCC 5376	161 ± 3	88 ± 4	70 ± 2	53 ± 5
ASCC 192	190 ± 4	135 ± 2	91 ± 3	55 ± 3

± Values refer to standard error of means
Values are means of triplicates from two separate runs, n = 6.
[1] Means viable count (CFU/g).

Table 3.4 Survival of twenty two different *Lactobacillus* strains during three hours at pH 3.0 in HCl solution

	Viable count (Log) cfu / g[1]			
Bacterial strain	0h	1h	2h	3h
ASCC 292	264 ± 4	149 ± 2	150 ± 3	140 ± 3
ASCC 290	144 ± 3	123 ± 5	129 ± 5	104 ± 5
ASCC 279	191 ± 8	145 ± 5	106 ± 2	134 ± 1
ASCC1521	161 ± 3	159 ± 5	134 ±4	131 ± 2
ASCC1520	161 ± 5	150 ±7	100 ± 2	144 ± 6
ATCC 15820	236 ± 2	195 ± 3	143 ± 2	122 ± 3
CSCC 2607	231 ± 7	215 ± 4	243 ± 6	248 ± 4
CSCC5203	189 ± 4	178 ± 6	170 ± 4	55 ± 6
ASCC295	192 ± 5	185 ± 4	183 ± 3	86 ± 4
ASCC 526	173 ± 4	152 ± 2	159 ± 3	42 ± 2
ASCC 1180	163 ± 4	101 ± 2	100 ± 3	89 ± 3
ASCC 277	186 ± 3	140 ± 2	115 ± 4	88 ± 2
ASCC 1002	160 ± 4	108 ± 2	89 ± 6	60 ± 1
ASCC1181	186 ± 6	80 ± 1	58 ± 2	40 ± 1
ASCC 1519	179 ± 5	132 ± 1	130 ± 4	35 ± 6
ASCC 1522	200 ± 2	180 ± 6	176 ± 4	55 ± 2
ASCC 1523	158 ± 3	109 ± 2	88 ± 4	72 ± 1
CSCC2603	215 ± 8	178 ± 6	170 ± 5	68 ± 6
CSCC 2605	222 ± 7	178 ± 1	165 ± 4	61 ± 6
CSCC 2627	159 ± 2	142 ± 6	140 ± 2	33 ± 6
CSCC 5376	142 ± 4	108 ± 4	88 ± 6	84 ± 2
ASCC 192	195 ± 3	165 ± 2	95 ± 1	75 ± 2

± Values refer to standard error of means
Values are means of triplicates from two separate runs, n = 6.
[1] Means viable count (CFU/g).

Table 3.5 Survival of twenty two different *Lactobacillus* strains during three hours in the presence of 0.0% bile solution

	Viable count (Log) cfu / g[1]			
Bacterial strain	0h	1h	2h	3h
ASCC 292	161 ± 3	175 ± 2	189 ±1	201 ± 3
ASCC 290	195 ± 5	199 ± 2	215 ± 3	255 ± 1
ASCC 279	169 ± 2	175 ± 2	190 ± 4	210 ± 2
ASCC1521	190 ± 3	185 ± 2	215 ± 3	269 ± 4
ASCC1520	159 ± 1	179 ± 4	190 ± 3	205 ± 6
ATCC 15820	210 ± 3	215 ± 3	230 ± 7	245 ± 6
CSCC 2607	180 ± 2	189 ± 4	200 ± 6	229 ± 4
CSCC5203	175 ± 3	192 ± 2	213 ± 4	250 ± 2
ASCC295	152 ± 2	169 ± 2	180 ± 6	195 ± 3
ASCC 526	139 ± 6	152 ± 5	163 ± 4	172 ± 6
ASCC 1180	110 ± 2	120 ± 4	140 ± 2	170 ± 6
ASCC 277	181 ± 3	195 ± 5	208 ± 4	215 ± 7
ASCC 1002	149 ± 7	165 ± 3	181 ± 4	201 ± 3
ASCC1181	188 ± 3	191 ± 5	210 ± 2	222 ± 8
ASCC 1519	160 ± 5	177 ± 3	192 ± 4	210 ± 7
ASCC 1522	118 ± 4	132 ± 3	155 ± 6	178 ± 2
ASCC 1523	151 ± 2	178 ± 4	194 ± 3	208 ± 5
CSCC2603	135 ± 7	158 ± 4	172 ± 8	199 ± 3
CSCC 2605	219 ± 5	230 ± 6	255 ± 6	285 ± 7
CSCC 2627	186 ± 6	195 ± 2	204 ± 3	228 ± 7
CSCC 5376	165 ± 3	180 ± 5	196 ± 4	211 ± 4
ASCC 192	185 ± 3	194 ± 5	204 ± 2	212 ± 8

± Values refer to standard error of means
Values are means of triplicates from two separate runs, n = 6.
[1] Means viable count (CFU/g).

Table 3.6 Survival of twenty two different *Lactobacillus* strains during three hours in the presence of 1.0% bile solution

	Viable count (Log) cfu / g[1]			
Bacterial strain	0h	1h	2h	3h
ASCC 292	268 ± 7	268 ± 7	168 ± 3	162 ± 3
ASCC 290	161 ± 2	154 ± 5	158 ± 6	157 ± 2
ASCC 279	164 ± 2	165 ± 2	159 ± 4	154 ± 6
ASCC1521	178 ± 4	178 ± 2	175 ± 4	173 ± 5
ASCC1520	159 ± 7	157 ± 4	151 ± 6	150 ± 1
ATCC 15820	221 ± 6	183 ± 1	189 ± 3	181 ± 2
CSCC 2607	228 ± 4	221 ± 6	223 ± 5	244 ± 9
CSCC5203	195 ± 6	180 ± 4	174 ± 3	150 ± 2
ASCC295	182 ± 4	168 ± 2	161 ± 4	63 ± 2
ASCC 526	163 ± 2	152 ± 3	140 ± 3	87 ± 5
ASCC 1180	181 ± 4	120 ± 1	92 ± 3	80 ± 1
ASCC 277	191 ±3	135 ± 4	103 ± 5	76 ± 2
ASCC 1002	179 ± 7	154 ± 7	99 ± 1	70 ± 2
ASCC1181	178 ± 3	135 ± 4	99 ± 1	80 ± 4
ASCC 1519	169 ±5	101 ± 3	119 ± 2	46 ± 2
ASCC 1522	218 ± 4	152 ± 3	159 ± 4	63 ± 1
ASCC 1523	171 ± 2	63 ± 6	52 ± 4	40 ± 2
CSCC2603	235 ± 7	160 ± 4	152 ± 5	35 ± 2
CSCC 2605	219 ± 5	92 ± 5	79 ± 1	56 ± 3
CSCC 2627	186 ± 6	132 ±3	122 ± 6	61 ± 4
CSCC 5376	161 ± 3	88 ± 4	70 ± 2	53 ± 5
ASCC 192	168 ± 3	155 ± 4	109 ± 1	90 ± 4

± Values refer to standard error of means
Values are means of triplicates from two separate runs, n = 6.
[1] Means viable count (CFU/g).

Table 3.7 Survival of twenty two different *Lactobacillus* strains during three hours in the presence of 1.5% bile solution

	Viable count (Log) cfu / g[1]			
Bacterial strain	0h	1h	2h	3h
ASCC 292	266 ± 7	58 ± 6	55 ± 9	39 ± 3
ASCC 290	116 ± 5	66 ± 1	35 ± 1	60 ± 2
ASCC 279	197 ± 7	109 ± 2	69 ± 2	45 ± 2
ASCC1521	126 ± 4	96 ± 2	84 ± 2	69 ± 1
ASCC1520	160 ± 6	106 ± 4	72 ± 3	43 ± 1
ATCC 15820	213 ± 9	164 ± 4	101 ± 2	51± 4
CSCC 2607	238 ± 7	145± 3	96 ± 2	47 ± 3
CSCC5203	163 ± 8	109 ± 2	88 ± 4	0
ASCC295	186 ± 4	155 ± 3	0	0
ASCC 526	204 ± 2	175 ± 6	90 ± 7	0
ASCC 1180	185 ± 4	125 ± 6	0	0
ASCC 277	190 ± 4	125 ± 5	52 ± 1	0
ASCC 1002	161 ± 3	0	0	0
ASCC1181	172 ± 4	102 ± 4	85 ± 1	0
ASCC 1519	193 ± 6	116 ± 6	63 ± 2	0
ASCC 1522	201 ± 7	100 ± 4	0	0
ASCC 1523	155 ± 6	0	0	0
CSCC2603	239 ± 9	160 ± 8	89 ± 4	0
CSCC 2605	227 ± 8	133 ± 4	0	0
CSCC 2627	190 ± 2	116 ± 6	0	0
CSCC 5376	178 ± 7	112 ± 8	63 ± 3	0
ASCC 192	165 ± 6	94 ± 2	0	0

± Values refer to standard error of means
Values are means of triplicates from two separate runs, n = 6.
[1] Means viable count (CFU/g).

Table 3.8 Deconjugation of sodium glycocholate and sodium taurocholate by *Lactobacillus* strains

Strains	Released cholic acid (mM)		
	Sodium glycocholate	Sodium taurocholate	Sodium glycocholate + sodium taurocholate
CSCC 2607	$1.80 \pm 0.11^{c, A}$	$1.41 \pm 0.15^{b, A}$	$1.35 \pm 0.38^{bc, A}$
ATCC 15820	$3.41 \pm 0.18^{a, A}$	$2.45 \pm 0.14^{ab, A}$	$2.11 \pm 0.30^{ac, A}$
ASCC 290	$1.92 \pm 0.50^{c, A}$	$1.20 \pm 0.14^{b, A}$	$1.68 \pm 0.14^{ac, A}$
ASCC 292	$2.30 \pm 0.40^{bc, A}$	$1.34 \pm 0.13^{b, A}$	$2.05 \pm 0.33^{ac, A}$
ASCC 1520	$1.58 \pm 0.14^{c, A}$	$1.29 \pm 0.11^{b, A}$	$1.16 \pm 0.09^{c, A}$
ASCC 1521	$4.70 \pm 0.44^{a, A}$	$3.29 \pm 0.38^{a, AB}$	$2.71 \pm 033^{ab, A}$
ASCC 279	$1.85 \pm 0.33^{c, A}$	$1.45 \pm 0.12^{b, A}$	$1.92 \pm 0.19^{ac, A}$

± values refer to standard error
Values are means of triplicates from two separate runs, n = 6.
^{abc} Means within a coloum with different lowercase letters are significantly different ($p< 0.05$)
^{AB} Means within a row with different uppercase letters are significantly different ($p< 0.05$)

3.5 Conclusion

Results obtained in our study have shown that all twenty two strains of *Lactobacillus* spp. showed good tolerance against acid and bile concentration. ASCC 1520, ASCC 1521, ASCC 279, ASCC 290, ASCC292, ATCC 15820 & CSCC 2607 have shown excellent acid and bile tolerance. So we selected all seven robust *Lactobacillus* strains for their deconjugation activity to further confirm their tolerance against bile in order to study further for their therapeutic benefits in this project.

4.0 VIABILITY AND PROTEOLYTIC ACTIVITY OF FREEZE-DRIED *LACTOBACILLUS* STRAINS AT VARIOUS TEMPRATURES DURING STORAGE

4.1 Introduction

The human gastrointestinal micorflora under normal circumstances is a stable ecosystem in which the microorganisms remain relatively constant, although there are certain interpersonal variations (Fuller, 1992). The role of the normal microflora is still poorly understood, but two of the most important functions from the host's point of view, appear to maintain resistance to colonization by infections caused by pathogens and perform certain metabolic functions (Fuller, 1989). Maintenance of the intestinal ecological flora is important in preventing pathogenic bacteria. A widespread prescription of antibiotics not only has led to an increase in antibiotic-resistant pathogens, but is often associated with the disruption of the protective flora leading to predisposition to infections. For these reasons, the control of infections through a nonantibiotic approach is urgently needed and bacterial replacement therapy using the natural flora is a promising alternative. Probiotic bacteria are live microorganisms belonging to the natural flora with no pathogenicity, but with functions of importance to the health and well being of the host (Bruno & Shah, 2003). It is increasingly accepted that these bacteria might represent effective tools for controlling overgrowth of pathogen and thereby preventing infections. Indeed, numerous in vitro and in vivo studies performed with different probiotics bacteria have shown the capabilities of these bacteria to interference with both growth and virulence properties (Arvola *et al.,* 1999; Cocconnier *et al.,* 1998).

The survival of probiotic bacteria in dairy products and viability of lactobacilli is important in providing a number of therapeutic benefits to consumers (Rybka & Kailasapathy, 1995). Consequently, many dairy products have now been reformulated to include *Lactobacillus* strains; they have now captured more than 7.5% yoghurt market in Australia (Australian Dairy Corporation, 1993). To provide health benefits, the suggested concentration for probiotic bacteria is 10^6 cfu/g of a product (Dave & Shah, 1997a; Robinson, 1987).

Probiotic cultures in food should be well defined and correctly and taxonomically named, available in a viable state in sufficient quantities for the entire period of shelf life, able to reach the intestine in a viable state and in sufficient numbers, able to benefit consumer, and genetically stable (Reuter *et al.,* 2002). However, the viable microbial count of many probiotic products has been questionable. Analysis of products has confirmed poor survival of probiotic bacteria in yoghurt and fermented milks (Shah, 2000).

Viability of lactobacilli in fermented products declines over time because of acidity of product, storage temperature, storage time and depletion of nutrients (Dave & Shah, 1997a). As a result, freeze dried probiotic bacteria are marketed as capsules or packaged in foil sachets. Similarly, fermented milk products containing *Lactobacillus* spp. and *Bifidobacterium* spp. have been developed. There is an increasing interest in dairy products containing specific bacterial species with potential health benefits (Portier *et al.,* 1993). However, slow growth of *Lactobacillus* spp. poses major difficulties for market expansion of probiotic products (Brasheras & Gilliland, 1995). Lactic acid bacteria (LAB) are characterised by their high demand for essential growth factors such as peptides and amino acids. However milk does not contain sufficient free amino acids and peptides to allow growth of LAB (Abu-Tarboush, 1996).

There is limited information available on viability and proteolytic activity of *Lactobacillus* spp. in probiotic products at various temperatures. A correct storage

temperature of probiotic preparation is essential to maintain viable population of freeze-dried probiotic bacteria. This study examined survival and proteolytic activity of 7 different freeze-dried *Lactobacillus* spp. at 3 different storage temperatures (-18^{0}C, 4^{0}C and 20^{0}C) during 12 months storage.

4.2 Material and Methods

4.2.1 Bacteria

Seven different *Lactobacillus* strains (ASCC 1520, ASCC 1521, CSCC 2607, ATCC 15820, ASCC 290, ASCC 292 and ASCC 279) were obtained from Australian Starter Culture Research Centre (Werribee, Australia). The organisms were grown in sterile de Mann, Rogosa, Sharpe (MRS) broth (Oxoid LTD., West Heidelberg, Australia) and were stored at -80^{0}C in 12% (wt/vl) sterile (121^{0}C for 15 min) reconstituted skim milk and glycerol (40% vol/vol).

4.2.2 Freeze-drying

Pure cultures of all seven strains were transferred from frozen stock cultures into MRS broth and incubated at 37^{0}C for 24 h and three successive transfers were carried out. The cultures were then centrifuged at 4500 × g at 4^{0}C for 15 min. The cell pellet was suspended in twenty milliliters of sterile 0.1M sodium phosphate buffer (pH 6.8) and re centrifuged (2714×g at 4^{0}C, 15 min). The cell pellet was suspended in sodium phosphate buffer (20 mL) containing Unipectine^{TM} RS 150 (Savannah Biosystems, Balwyn East, Australia) at 2% (wt/vol) as a cryoprotectant. The cell suspension was then aseptically poured into large petri dishes, sealed with paraffin and aluminum foil, and frozen overnight at -18^{0}C, followed by freeze-drying for 48 h using a freeze-dryer (model FD-300. Airvac Engineering Pty Ltd., Dandenong, Australia). The freeze dryer was programmed to operate for 10 min of initial freezing after internal pressure was reduced to -100 Torr, 44 h of primary freezing, and 4 h of secondary freezing. The temperature was maintained at -88^{0}C. Freeze-dried cells were stored in sterile, sealed, plastic bags. An initial bacterial count was performed using MRS agar at 37^{0}C for 48 h. They were then stored separately in sealed plastic bags in a freezer at -18^{0}C in

a refrigerator at 4^0C, and at room temperature (approximately 20^0C) for enumeration of viable population and proteolytic activity.

4.2.3Enumeration of freeze-dried lactobacilli

Enumeration of all seven strains was performed in duplicate at 0, 1, 2, 4, 6, 9 and 12 mo of storage. The pour plate method was used for enumeration of the viable population of Lactobacilli. MRS agar was used for enumeration and the plates were incubated at 37^0C for 72 h. Counts were counted as colony forming units (CFU) per milliliter.

4.2.4 Proteolytic activity in reconstituted skim milk

One gram of all freeze-dried *Lactobacillus* strains were grown overnight at 37^0C in MRS broth. To minimize carryover of free amino acids during inoculation, 5 mL of cells were washed and re-suspended to the original volume with sterile 0.32 mM sodium phosphate, pH 7.2. Cells were inoculated (1%) into RSM 12% (w/v) and incubated at 37^0C for 6 h. A control consisted of un inoculated RSM. A 2.5 mL sample of each incubated RSM was mixed with 10 mL of 0.75M (7.7%) trichloroacetic acid (TCA) and 1 mL of water to 5 mL of sample to give a final concentration of 0.47 M (7.7%) TCA. The samples were filtered using Whatman number 4A filter paper after 10 min incubation at room temperature (~22^0C) and frozen until assayed. The *o*-phthaldialdehyde (OPA) method described by Church *et al.* (1983) was used to determine the concentration of free amino groups in the filtrate. Triplicate aliquots from each TCA filtrate were analysed using a Pharmacia LKB Novaspek II spectrophotometer (Pharmacia, Biotech, Uppsala, Sweden) at 340 nm.

4.2.5 Statistical analysis

The counts of freeze dried bacteria stored at -18^0C, 4^0C and 20^0C were analyzed. All enumerations were performed in duplicate, and data were presented in mean $\log_{10}$ ± standard error (n = 6). Analysis of variance (ANOVA) was performed using the software package decision tools suite software StatPro^{TM} for Microsoft Excel (N.Y., U.S.A). ANOVA was used

to test between different storage temperatures for each product where $p < 0.05$ was considered statistically significant.

4.3 Results and discussions

4.3.1 Proteolytic activity

Table 4.1 represents the proteolytic activity of 7 different freeze dried *Lactobacillus* strains stored up to 12 mo at -18^{0}C. Samples were taken at (0, 1, 2, 4, 6, 9 and 12) monthly intervals. The proteolytic activity of these bacterial cultures is expressed as the amount of free amino groups measured as difference in absorbance values at 340 nm, after subtraction of values for the uninoculated control RSM. The extent of proteolysis based upon OPA values were variable between 0.22 to 0.15 at 0 mo, 0.24 to 0.16 at 1 mo, 0.23 to 0.15 at 2 mo, 0.21 to 0.14 at 4 mo., 0.22 to 0.15 at 6 mo, 0.23 to 0.14 at 9 mo and 0.25 to 0.15. *L. rhamnosus* 1520, 2607 and *L. paracasei* 279 have shown higher proteolytic activity compare to *L. rhamnosus* 1521, *L. zeae* 15820, *L. casei* 290 and *L. paracasei* 292 respectively.

The OPA-based spectrophotometric assay is very good method for detecting released α-amino groups, which result from the proteolysis of milk proteins and gives a direct measurement of proteolytic activity. Results obtained from these experiments shows that freeze drying activity is not affecting proteolytic activity of *Lactobacillus* group. Starter cultures containing *L. acidophilus*, *Bifidobacterium* spp., *L. casei* and *S. thermophilus* (ABCT- cultures) are becoming popular around world. ABT-cultures lack *L. delbrueckii* ssp. *Bulgaricus,* and as a result, the fermentation time for yoghurt making is longer with these cultures (Dave & shah, 1997). During this experiment Lactobacilli had shown generally higher proteolytic activity than *L. acidophilus* and *Bifidobactrium* spp. (Shihata & shah 2000).

4.3.2 Freeze drying activity

L. rhamnosus 1520, 2607 and 1521 in freeze dried preparations stored for up to 12 mo at-18^{0}C, 4^{0}C and 20^{0}C. Samples were taken at regular intervals for viable counts are presented in Tables 4.2a, 4.2b and 4.2c respectively. The viable counts of *L. rhamnosus* 1520

decreased ($p < 0.05$) from 9.60 to 8.22, *L. rhamnosus* 2607 10.65 to 8.64 and *L. rhamnosus* 1521 10.82 to 8.91 log CFU/mL over the 12 mo of storage at -18^{0}C respectively. The same strain stored at 4^{0}C over 12 mo revealed a higher decrease ($p < 0.05$) in viability and a greater drop in count from 9.60 to 7.25, 10.65 to 7.77 and 10.82 to 7.45 log CFU/mL respectively. All strains of freeze dried *L. rhamnosus* counts stored at 20^{0}C decreased significantly ($p < 0.05$), and no organisms were detected after 2 mo.

The counts of freeze dried *L. casei* 290 and *L. zeae* 15820 stored at -18^{0}C, 4^{0}C and 20^{0}Chave shown in Tables 4.2d and 4.2e. The viable counts of *L. casei* 290 and *L. zeae* 15820 were decreased ($p < 0.05$) 10.15 to 9.01 and 9.98 to 8.04 log CFU/mL over the 12 mo of storage at -18^{0}C. The same strains stored at 4^{0}C for 12 mo exhibited a larger decrease ($p < 0.05$) in viability from 10.15 to 7.51 and 9.98 to 7.10 log CFU/mL. The freeze dried *Lactobacillus* counts stored at 20^{0}C decreased significantly ($p < 0.05$), and no organisms were detected after 2 mo.

Freeze dried *L. paracasei* 292 and 279 stored for up to 12 mo at -18^{0}C, 4^{0}C and 20^{0}C are presented in tables 2f and 2g. Samples were taken at regular intervals as shown in tables. The counts of *L. paracasei* 292 and 279 were decreased significantly ($p < 0.05$) 10.1 to 8.25 and 9.95 to 8.15 log CFU/mL over the 12 mo of storage at -18^{0}C. The same strains stored at 4^{0}C for 12 mo revealed a decrease ($p < 0.05$) in viability and a greater drop in count from 10.1 to 7.81 and 9.95 to 7.58 log CFU/mL. The freeze dried *L. paracasei* counts stored at 20^{0}C decreased significantly ($p < 0.05$), and no organisms were detected after 2 mo.

The loss of viability after four months of storage at room temperature is possibly due to increase of metabolic and cellular activity within the all seven strains at 20^{0}C that may have led to exhaustion of nutrients stored within the cell. The microorganisms that were stored at frozen temperatures had maximum bacterial counts due to their lack of metabolic and cellular activity; therefore, any loss of viability was due to cells that were affected by freezing.

Freeze-dried probiotic microorganisms that were stored at refrigerated conditions had lower viability than those stored at frozen temperature due to slight metabolic and cellular activity at the refrigerated temperature.

4.4 Tables

Table 4.1 Proteolytic activity of all bacterial strains during storage at -18^{0}C[1]

	OD at 340 nm						
Probiotic strains	0 Month	1 Month	2 Months	4 Months	6 Months	9 Months	12 Months
ASCC 1520	0.26 ± 0.01	0.24 ± 0.04	0.23 ± 0.01	0.21 ± 0.06	0.22 ± 0.04	0.20 ± 0.04	0.22 ± 0.05
ASCC 1521	0.15 ± 0.03	0.17 ± 0.01	0.16 ± 0.02	0.17 ± 0.02	0.16 ± 0.02	0.18 ± 0.04	0.15 ± 0.01
CSCC 2607	0.24 ± 0.02	0.25 ± 0.03	0.22 ± 0.01	0.21 ± 0.05	0.23 ± 0.01	0.22 ± 0.05	0.24 ± 0.07
ATCC 15820	0.16 ± 0.01	0.18 ± 0.02	0.16 ± 0.03	0.14 ± 0.02	0.19 ± 0.08	0.14 ± 0.06	0.17 ± 0.05
ASCC 290	0.17 ± 0.04	0.14 ± 0.08	0.17 ± 0.02	0.19 ± 0.04	0.20 ± 0.06	0.18 ± 0.02	0.17 ± 0.01
ASCC 292	0.15 ± 0.01	0.16 ± 0.04	0.15 ± 0.01	0.17 ± 0.02	0.15 ± 0.01	0.19 ± 0.04	0.18 ± 0.09
ASCC 279	0.25 ± 0.02	0.21 ± 0.01	0.22 ± 0.01	0.23 ± 0.03	0.20 ± 0.05	0.23 ± 0.04	0.25 ± 0.2

[1] Indication of proteolytic activity of Lactobacilli strains after incubation in RSM at 37^{0}C for 6h.
Data represent differences in absorbance values after subtracting the value (1.68) for the control RSM.

Table 4.2a Viable microbial count (log_{10} colony-forming units (cfu)/mL) of freeze dried *L. rhamnosus*1520 stored for 12 mo (mean ± standard error)

Sampling time (mo)	Log_{10} CFU/mL[a,b,e]		
	Frozen (-18^0C)	Refrigerated (4^0C)	Room temp. (20^0C)
0[c]	9.60 ± 0.01a	9.60 ± 0.01a	9.60 ± 0.01a
1	9.12 ± 0.04a	9.05 ± 0.02a	7.25 ± 0.02b
2	8.89 ± 0.01a	8.21 ± 0.01b	5.88 ± 0.04c
4	8.66 ± 0.02a	7.90 ± 0.05b	ND[d]
6	8.51 ± 0.04a	7.81 ± 0.1b	ND
9	8.45 ± 0.06a	7.77 ± 0.07b	ND
12	8.22 ± 0.02a	7.25 ± 0.21b	ND

[a] Mean standard error (n=6), [b] Means the same row followed by different lower case letters are significantly different ($p<0.05$). [C] 0 mo= initial counts immediately after freeze drying. [d] ND = not detectable. [e]One way analysis of variance of means in the same row ($\alpha= 0.05$)

Table 4.2b Viable microbial count (log_{10} colony-forming units (cfu)/mL) of freeze dried *L. rhamnosus*2607 stored for 12 mo (mean ± standard error)

Sampling time (mo)	Log_{10} CFU/mL[a,b,e]		
	Frozen (-18^0C)	Refrigerated (4^0C)	Room temp. (20^0C)
0[c]	10.65 ± 0.1a	10.65 ± 0.1a	10.65 ± 0.1a
1	10.24 ± 0.01a	9.97 ± 0.03b	9.84 ± 0.04b
2	9.81 ± 0.02a	9.15 ± 0.04b	7.22 ± 0.02c
4	9.65 ± 0.03a	8.89 ± 0.01b	ND[d]
6	9.15 ± 0.03a	8.51 ± 0.02b	ND
9	8.91 ± 0.04a	8.12 ± 0.05b	ND
12	8.64 ± 0.01a	7.77 ± 0.05b	ND

[a] Mean standard error (n=6), [b] Means the same row followed by different lower case letters are significantly different. ($p<0.05$). [C] 0 mo= initial counts immediately after freeze drying. [d] ND = not detectable. [e]One way analysis of variance of means in the same row ($\alpha= 0.05$)

Table 4.2c Viable microbial count (log_{10} colony-forming units (cfu)/mL) of freeze dried *L. rhamnosus* 1521 stored for 12 mo (mean ± standard error)

Sampling time (mo)	Log_{10} CFU/mL[a,b,e]		
	Frozen (-18^0C)	Refrigerated (4^0C)	Room temp. (20^0C)
0[c]	10.82 ± 0.08a	10.82 ± 0.08a	10.82 ± 0.08a
1	10.25 ± 0.02a	9.94 ± 0.01b	9.55 ±0.02b
2	9.80 ± 0.01a	9.40 ± 0.02b	6.88 ± 0.03c
4	9.65 ± 0.02a	9.08 ± 0.01b	ND[d]
6	9.41 ± 0.01a	8.85 ± 0.04b	ND
9	9.11 ± 0.04a	8.01 ± 0.02b	ND
12	8.91 ± 0.02a	7.45 ± 0.05b	ND

[a] Mean standard error (n=6), [b] Means the same row followed by different lower case letters
are significantly different. ($p<0.05$). [C] 0 mo= initial counts immediately after freeze drying.
[d] ND = not detectable. [e]One way analysis of variance of means in the same row ($\alpha= 0.05$)

Table 4.2d Viable microbial count (log_{10} colony-forming units (cfu)/mL) of freeze dried *L. casei* 290 stored for 12 mo (mean ± standard error)

	Log_{10} CFU/mL[a,b,e]		
Sampling time (mo)	Frozen (-18^0C)	Refrigerated (4^0C)	Room temp. (20^0C)
0[c]	10.15 ±0.02a	10.15 ±0.02a	10.15 ±0.02a
1	9.95 ± 0.01a	9.64 ± 0.04b	9.78 ± 0.01b
2	9.81 ± 0.08a	9.44 ± 0.01b	7.15 ± 0.04c
4	9.71 ± 0.01a	9.11 ± 0.04b	ND[d]
6	9.37 ± 0.02a	8.77 ± 0.01b	ND
9	9.15 ± 0.1a	7.95 ± 0.05b	ND
12	9.01 ± 0.03a	7.51 ± 0.06b	ND

[a] Mean standard error (n=6), [b] Means the same row followed by different lower case letters are significantly different. ($p<0.05$). [C] 0 mo= initial counts immediately after freeze drying.

[d] ND = not detectable. [e]One way analysis of variance of means in the same row ($\alpha= 0.05$)

Table 4.2e Viable microbial count (log_{10} colony-forming units (cfu)/mL) of freeze dried *L.zeae* 15820 stored for 12 mo (mean ± standard error)

Sampling time (mo)	Log_{10} CFU/mL[a,b,e]		
	Frozen (-18^0C)	Refrigerated (4^0C)	Room temp. (20^0C)
0[c]	9.98 ± 0.2a	9.98 ± 0.2a	9.98 ± 0.2a
1	9.85 ± 0.01a	9.75 ± 0.05b	8.10 ±0.06c
2	9.60 ± 0.05a	9.40 ± 0.04b	7.52 ± 0.01c
4	9.18 ± 0.04a	8.77 ± 0.01b	ND[d]
6	8.95 ±0.05a	8.10 ± 0.06b	ND
9	8.55 ± 0.01a	7.95 ± 0.02b	ND
12	8.04 ± 0.1a	7.10 ± 0.04b	ND

[a] Mean standard error (n=6), [b] Means the same row followed by different lower case letters are significantly different. ($p<0.05$). [C] 0 mo= initial counts immediately after freeze drying.
[d] ND = not detectable. [e]One way analysis of variance of means in the same row ($\alpha= 0.05$)

Table 4.2f Viable microbial count (log_{10} colony-forming units (cfu)/mL) of freeze dried *L. paracasei* 292stored for 12 mo (mean ± standard error)

Sampling time (mo)	Log_{10} CFU/mL[a,b,e]		
	Frozen (-18^0C)	Refrigerated (4^0C)	Room temp. (20^0C)
0[c]	10.1 ± 0.05a	10.1 ± 0.05a	10.1 ± 0.05a
1	9.98 ± 0.02a	9.85 0.02b	9.56 ± 0.02c
2	9.80 ± 0.01a	9.74 ± 0.03b	6.88 ± 0.01c
4	9.64 ± 0.03a	9.10 ± 0.02b	ND[d]
6	9.25 ± 0.08a	8.84 ± 0.4b	ND
9	8.94 ± 0.01a	8.17 ± 0.1b	ND
12	8.25 ± 0.04a	7.81 ± 0.09b	ND

[a] Mean standard error (n=6), [b] Means the same row followed by different lower case letters
are significantly different. ($p<0.05$). [C] 0 mo= initial counts immediately after freeze drying.
[d] ND = not detectable. [e]One way analysis of variance of means in the same row ($\alpha= 0.05$)

Table 4.2g Viable microbial count (log_{10} colony-forming units (cfu)/mL) of freeze dried *L. paracasei* 279 stored for 12 mo (mean ± standard error)

Sampling time (mo)	Log_{10} CFU/mL[a,b,c,d]		
	Frozen (-18^0C)	Refrigerated (4^0C)	Shelf (20^0C)
0[c]	9.95 ± 0.06a	9.95 ± 0.06a	9.95 ± 0.06a
1	9.78 ± 0.01a	9.68 ± 0.02b	8.40 ± 0.02c
2	9.41 ± 0.2a	9.49 ± 0.04b	6.48 ± 0.01c
4	9.16 ± 0.05a	8.77 ± 0.2b	ND[d]
6	8.80 ± 0.04a	8.41 ± 0.1b	ND
9	8.50 ± 0.02a	7.94 ± 0.04b	ND
12	8.15 ± 0.05a	7.58 ± 0.01b	ND

[a] Mean standard error (n=6), [b] Means the same row followed by different lower case letters
are significantly different. ($p<0.05$). [C] 0 mo= initial counts immediately after freeze drying.
[d] ND = not detectable. [e]One way analysis of variance of means in the same row ($\alpha= 0.05$)

4.5 Conclusions

This study has established that a temperature maintained at -18^{0}C was ideal for the long term storage for *Lactobacillus* strains. All 7 *Lactobacillus* strains provided maximum viability and proteolytic activity. The bacterial counts reduced at refrigerated temperatures (4^{0}C), whereas storage temperature of 20^{0}C reduced the viable count significantly. Similarly all *Lactobacillus* strains stored at -18^{0}C shown higher proteolytic activity up to 12 mo of storage. These results gave promising indication of future commercial use of *Lactobacillus* strains either in pharmaceutical or cultured milk products.

5.0 SURVIVAL AND ACTIVITY OF PROBIOTIC LACTOBACILLI IN SKIM MILK CONTAINING PREBIOTICS

5.1 Introduction

Lactobacilli are typically characterized as Gram-positive, non-spore forming, non-motile, catalase-negative bacteria growing under microaerobic or strictly anaerobic conditions. Some species produce lactic and acetic acids when they use glucose as carbon source (Hammes and others 1995). Lactobacilli are claimed to provide a number of health benefits including antimicrobial effects against pathogenic bacteria, anti-tumor effects, and protection against antibiotic-associated diarrhea or food allergy (Saxelin 1997; Orrhage and Nord 2000; Cummings and others 2001). Lactobacilli are reported to be acid and bile tolerant and survive in the gastrointestinal tract.

The human colon is one of the body's most metabolically active organs. Gut bacteria predominantly ferment undigested food materials. This nature of fermentation may have different health consequences. There has been an increasing interest in use of diet to fortify certain gut flora (Fooks and others 1999). In this context probiotics and prebiotics play significant roles. Probiotics are live microorganisms which when added to foods help restore gut flora of the host. Prebiotics are non-digestible food ingredients, which are beneficial to the gut microflora.

[1] A version of this chapter has been published: Desai, A. R., I. B. Powell and N. P. Shah 2004. Survival and activity of probiotic lactobacilli in skim milk containing prebiotics *J Food Sci.* 69: 57-60.

'Synbiotics' are the combination of both approaches (Gibson and others 2000). Gibson and Roberfroid (1995) first introduced the term prebiotic, which is 'a non digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon' (Gibson and others 2000; Coussement 1996).

Large and small intestines have at least 10^{11} of bacteria per gram of gut content (Fooks and others 1999). Recent reports have confirmed the ability of probiotic bacteria to ferment oligosaccharides that bypass mammalian metabolism (Kaplan and Hutkins 2000; Gibson and others 2000). Oligosaccharides are a group of short chain non-digestible polysaccharides that occur naturally in foods (Crittenden 1999). Commercially, enzymatic processes produce oligosaccharides either by hydrolysis of polysaccharides or synthesis from smaller sugars using transglycosylases (Bruno and others 2002). There has been a considerable interest in the use of prebiotics to enhance the survival and colonization of probiotic bacteria that are added in food products (Ziemer and Gibson 1998). A range of oligosaccharides has been tested using various methods to assess their prebiotic effect (Gibson and others 2000; Kalpan and Hutkins 2000; Roberfroid 2001). Some research with prebiotics has shown reductions in putative risk factors for colon cancer (Parody 1999) and control of serum triglycerides and cholesterol (Roberfroid 2001).

Lactobacilli have been used widely in dairy products due to their health-promoting effects (Kaplan and Hutkins 2000). Although the effect of oligosaccharides on colonic lactobacilli has not been studied widely, there are reports on the effects of oligosaccharides on lactobacilli in general. Therefore, the objective of this study was to investigate the effect of commercially available oligosaccharides and inulin on survival and activity of seven different strains representing *Lactobacillus casei* and related taxa (Dellaglio and others 2002).

5.2 Materials and Methods

5.2.1 Culture preparation

Seven *Lactobacillus* strains were obtained from the Australian Starter Culture Collection (Werribee, Australia), CSIRO Starter Culture Collection (Werribee, Australia) and American Type Culture Collection (Manassas, VA, USA). Each strain was cultured in MRS broth (Oxoid, Basingstoke, U.K.) containing 5% (w/v) lactose (MRSL) at 37^0C for 48 h. The cells were centrifuged for 15 min at 1000 x g at 4^0C and resuspended in pasteurised (63^0C for 30 min) 12% reconstituted skim milk (RSM).

5.2.2 Growth of lactobacilli in the presence of prebiotics

Commercially available Hi-maize (Starch Australasia Ltd., Lane Cove, Australia), raftilose (Orafti Pty. Ltd., Tienen, Belgium), lactulose and inulin (Sigma Chemical Co., St. Louis, MO, U.S.A.) were added at the rate of 5% (w/v) to 12% RSM in 250 mL glass bottles and pasteurised at 70^0C for 15 min. Control samples did not contain any prebiotics. Duplicate bottles of each treatment were prepared. Pasteurised RSM was inoculated (5% v/v) separately with each of seven *Lactobacillus* strains and incubated at 37^0C for 48 h. An aliquot from each treatment was taken at 0 h and 48 h and diluted (1:10, v/v) with 0.2% (w/v) EDTA (pH 12.0) and turbidity measured at 640 nm using a Novaspec II spectrophotometer (Pharmacia Biotech, LKB Biotech, Uppsala, Sweden). Uninoculated pasteurised RSM diluted with 0.2% (w/v) EDTA (pH 12.0) was used as a blank. Specific growth rate (μ) for each culture was calculated using the following equation: $\mu = (\ln X_2 - \ln X_1) / (t_2 - t_1)$, where X_2 and X_1 are the cell densities at times t_2 and t_1. Mean doubling time (T_d) was calculated as $T_d = \ln 2 / \mu$ (Shin and others 2000).

5.2.3 Activity determination

The activity of each culture in the presence of different prebiotics was determined by measuring end products of fermentation (lactic and acetic acids) using high performance liquid chromatography (HPLC, Varian Australia Ltd, Mulgrave, Australia). RSM containing 5% (w/v) inulin, Hi-maize, lactulose or raftilose and fermented by different *Lactobacillus* strains as described above were prepared for HPLC analysis using the method described by Dubey and Mistry (1996). One-hundred microlitres of 15.8 M HNO_3 and 14.9 mL of 9 mM H_2SO_4 were added to 1.5 mL of sample and centrifuged at 4000 x g for 10 min using a bench top centrifuge (Sorvall RT7, Millipore Corp., Bedford, MA, U.S.A.) and 2 mL aliquots were stored at -20^0C until analysed. An Aminex HPX-87H column with disposable cartridges H^+ (Bio-Rad Laboratories, Hercules, CA, USA) maintained at 65^0C was used for organic acid analysis. The degassed mobile phase of 9 mM H_2SO_4, filtered through a 0.45μm membrane filter (Millipore), was used at a flow rate of 0.3 mL/ min. The wavelength of detection was optimized at 220nm for the organic acids being quantified. Standard solutions of organic acids (lactic and acetic acids; Sigma) were prepared in water to establish elution times and calibration curves.

5.2.4 Determination of viability during refrigerated storage

Lactobacillus strains were cultured at 37^0C with 5% (w/v) of each of the prebiotics. Cultures containing no prebiotics were used as controls. All cultures were incubated at 37^0C for 48 h, and then stored at 4.0 ± 1^0C for 4 weeks. One milliliter of each fermented milk sample was diluted with 9 mL of sterile 0.1% (w/v) peptone water (Amyl Media Pty. Ltd., Dandenong, Australia) and mixed uniformly with a vortex mixer. Serial dilutions were prepared and viable numbers enumerated using the pour plate technique using MRS agar (Oxoid). Plates were

incubated at 37^0C for 48 h. Percent viabilities of each culture in the presence of each prebiotic were calculated as follows: % viability = (CFU at 4 weeks of storage / initial CFU) × 100 (Bruno and others 2002).

5.2.5 Determination of pH

The pH of samples was measured at 20 to 22^0C using a pH meter (Model 8417, Hanna Instruments Pty. Ltd., Singapore) after calibration performed with fresh pH 7.0 and 4.0 standard buffers.

5.2.6 Statistical Analysis

Each experiment was independently replicated three times in a completely randomized design. All analysis and enumerations were done in duplicate. Statistical analysis was conducted using Student's t-test.

5.3 Results and Discussions

The mean doubling time in hours of each of seven *Lactobacillus* strains grown in RSM in the presence of 5% of each of inulin, lactulose, raftilose and Hi-maize is shown in Table 5.1. Mean doubling time was used as a measure of the effectiveness of the oligosaccharides in modulating growth rate. In general, doubling time of *Lactobacillus* strains grown with prebiotics decreased as compared to the control. Mean doubling times were significantly lower ($p<0.05$) for all cultures containing 5% inulin, lactulose, Hi-maize and raftilose except ASCC 292 and ATCC 15820 with raftilose. Among the prebiotics tested, Hi-maize was most effective in enhancing growth rate of CSCC 2607 in RSM with doubling time of 301 min. All seven strains showed the fastest growth rate with Hi-maize. The highest doubling time recorded with prebiotics was 722 min by ATCC 15820 with raftilose.

Table 5.2 shows acetic and lactic acids produced by seven *Lactobacillus* strains grown in RSM containing 5% of each of the four prebiotics. The highest level of lactic acid was produced by ATCC 15820 with Hi-maize (1882 mg/L) and the lowest by ASCC 292 with raftilose (963 mg/L). The highest level of acetic acid produced by ATCC 15820 with lactulose (130.6 mg/L) while the lowest by CSCC 2607 with Hi-maize (31.3 mg/L). Lactic acid concentration produced by all seven strains in the presence of inulin and Hi-maize were significantly different from controls ($p<0.1$).

The viability of the seven strains grown in RSM containing probiotics after four weeks of storage at 4^0C is shown in Table5.3. In general, the viability of all seven strains decreased during storage. However, the viability was in many cases significantly higher ($p<0.05$) than that of control, without prebiotics. On an average best viability was observed with inulin. The highest

viability of 83% was recorded for CSCC 2607 with inulin. Overall raftilose and lactulose were the least effective in maintaining viability, with average viabilities of 44% and 47%. The lowest viability was recorded by ASCC 1521 with an average viability of 35%. The control samples containing no prebiotics had average survival rate of 33%. In our study, 35 to 83% viability was observed after 4 weeks of storage at 4^0C when prebiotics were added.

Table 5.4 shows pH changes in RSM during 4 weeks of storage. Due to lactic and acetic acid production by *Lactobacillus* strains, pH of all samples decreased. The pH values varied from 4.34 (ASCC 1520 with raftilose) to 4.10 (ATCC 15820 with inulin and ASCC 1520 with Hi-maize). Only inulin and Hi-maize significantly affect ($p<0.05$) the pH values.

5.4 Tables

Table 5.1. Doubling time of seven *Lactobacillus* strains in skim milk containing prebiotics[b]

	Mean doubling time (min)				
Prebiotic	Control	Inulin	Lactulose	Raftilose	Hi-maize
Strains					
ASCC 1520	597 ±45	437 ± 31[a]	428 ± 36[a]	488 ± 18[a]	367 ± 43[a]
ASCC 1521	711 ± 46	454 ± 25[a]	525 ± 56[a]	517 ± 32[a]	387 ± 42[a]
CSCC 2607	561 ± 29	358 ± 15[a]	380 ± 15[a]	420 ± 28[a]	301 ± 26[a]
ATCC 15820	751 ± 31	518 ± 48[a]	596 ± 84[a]	722 ± 58	500 ± 19[a]
ASCC 290	693 ± 58	393 ± 27[a]	455 ± 43[a]	489 ± 33[a]	357 ± 44[a]
ASCC 292	601 ± 52	457 ± 44[a]	467 ± 45[a]	549 ± 24	421 ± 52[a]
ASCC 279	622 ±31	412 ± 37[a]	382 ± 4[a]	511 ± 27[a]	337 ± 44[a]

[a] Significantly different ($p<0.05$) from the control.

[b] Measurements are means ± standard deviations of 3 replicates for all treatments.

Table 5.2. Acetic and lactic acid production by seven *Lactobacillus* strains grown in skim milk containing 5% prebiotics[b]

Prebiotic		Control	Inulin	Lactulose	Raftilose	Hi-maize
Strains						
ASCC 1520	Acetic acid (mg/L)	51.7 ± 1.7	44.8 ± 2.2[a]	34.6 ± 0.3[a]	43.0 ± 3.30[a]	40.8 ± 3.0[a]
	Lactic acid (mg/L)	1457 ± 5	1589 ± 14[a]	1579 ± 16[a]	1511 ± 15[a]	1506 ± 30[a]
ASCC 1521	Acetic acid (mg/L)	53.4 ± 1.0	49.1 ± 2.5	48.4 ± 2.2	65.2 ± 3.8[a]	50.9 ± 2.7
	Lactic acid (mg/L)	1438 ± 73	1529 ± 52[a]	1183 ± 45[a]	1274 ± 20[a]	1491 ± 43[a]
CSCC 2607	Acetic acid (mg/L)	31.1 ± 0.5	32.4 ± 1	31.6 ± 0.5	48.3 ± 3.6[a]	31.3 ± 0.5
	Lactic acid (mg/L)	1464 ± 30	1545 ± 3[a]	1606 ± 12[a]	1557 ± 10[a]	1607 ± 15[a]
ATCC 15820	Acetic acid (mg/L)	117.6 ± 4.8	114.4 ± 4.9	130.6 ± 3.5[a]	115.6 ± 3.6	113.8 ± 4.4
	Lactic acid (mg/L)	1350 ± 26	1484 ± 65[a]	1450 ± 56[a]	1392 ± 35	1882 ± 31[a]
ASCC 290	Acetic acid (mg/L)	76.4 ± 2.1	69.8 ± 4	76.7 ± 2.8	48.9 ± 3.6[a]	87.6 ± 4.3[a]
	Lactic acid (mg/L)	1439 ± 73	1530 ± 53[a]	1428 ± 27	1718 ± 47[a]	1827 ± 46[a]
ASCC 292	Acetic acid (mg/L)	105.9 ± 3.1	121 ± 1.7[a]	105.7 ± 2.6	108.3 ± 4	108.7 ± 1.8
	Lactic acid (mg/L)	1056 ± 28	1169 ± 32[a]	1281 ± 32[a]	963 ± 10[a]	1179 ± 10[a]
ASCC 279	Acetic acid (mg/L)	97.6 ± 2.1	78.7 ± 4.3[a]	100.5 ± 2.3	68.4 ± 1.6[a]	108.9 ± 5.3
	Lactic acid (mg/L)	1245 ± 21	1564 ± 23[a]	1334 ± 54[a]	1542 ± 28[a]	1373 ± 13[a]
Average	Acetic acid (mg/L)	78.1 ± 8	72.6 ± 2.6	75.4 ± 5.1	70.5 ± 9.1[a]	77.4 ± 4.8
	Lactic acid (mg/L)	1379 ± 50	1487± 60[a]	1408 ± 35	1422 ± 23[a]	1552 ± 50[a]

[a] Significantly different ($p<0.1$) from the control.

[b] Measurements are means ± standard deviations of 3 replicates for all treatments.

Table 5.3. Viability of *Lactobacillus* strains grown in skim milk containing prebiotics after 4 weeks of refrigerated storage at 4^0C [b]

Prebiotics Strains	Reading interval	Control	Inulin	Lactulose	Raftilose	Hi-maize
	0 wk	1.89×10^8	1.90×10^8	1.98×10^8	1.92×10^8	1.80×10^8
ASCC 1520	4 wk	6.52×10^7	9.10×10^7	8.56×10^7	8.60×10^7	8.50×10^7
	% Viability [c]	34.5 ± 0.1	52.1 ± 0.2[a]	43.2 ± 0.2[a]	44.8 ± 0.2[a]	47.2 ± 0.3[a]
	0 wk	1.89×10^8	1.99×10^8	1.85×10^8	1.75×10^8	2.05×10^8
ASCC 1521	4 wk	5.62×10^7	1.46×10^8	6.45×10^7	7.92×10^7	1.20×10^8
	% Viability [c]	29.7 ± 0.16	73.3 ± 0.3[a]	34.9 ± 0.2	45.3 ± 0.3[a]	58.5 ± 0.3[a]
	0 wk	1.65×10^8	2.00×10^8	1.99×10^8	1.85×10^8	1.99×10^8
CSCC 2607	4 wk	7.54×10^7	1.65×10^8	9.97×10^7	9.48×10^7	1.32×10^8
	% Viability [c]	33.3 ± 0.1	83 ± 0.2[a]	50 ± 0.3[a]	51.2 ± 0.2[a]	66.6 ± 0.2[a]
	0 wk	1.78×10^8	1.93×10^8	1.87×10^8	1.65×10^8	2.10×10^8
ATCC 15820	4 wk	5.08×10^7	1.25×10^8	1.12×10^8	9.99×10^7	1.45×10^8
	% Viability [c]	28.5 ± 0.2	64.7 ± 0.3[a]	59.8 ± 0.2[a]	60.5 ± 0.2[a]	69 ± 0.2[a]
	0 wk	1.65×10^8	1.90×10^8	1.75×10^8	1.89×10^8	1.74×10^8
ASCC 290	4 wk	6.87×10^7	1.11×10^8	7.45×10^7	8.78×10^7	1.19×10^8
	% Viability [c]	41.6 ± 0.3	58.4 ± 0.2[a]	42.5 ± 0.6[a]	46.4 ± 0.2	68.4 ± 0.3[a]
	0 wk	1.78×10^8	1.94×10^8	1.73×10^8	1.69×10^8	2.07×10^8
ASCC 292	4 wk	6.65×10^7	1.25×10^8	8.53×10^7	9.27×10^7	1.50×10^8
	% Viability [c]	37.4 ± 0.3	64.4 ± 0.3[a]	49.3 ± 0.5[a]	54.8 ± 0.2[a]	72.4 ± 0.4[a]
	0 wk	1.98×10^8	1.98×10^8	1.69×10^8	1.78×10^8	1.87×10^8
ASCC 279	4 wk	5.65×10^7	1.50×108	1.12×10^8	7.53×10^7	1.12×10^8
	% Viability [c]	28.5 ± 0.2	75.7 ± 0.3[a]	66.2 ± 0.2[a]	42.3 ± 0.6[a]	59.8 ± 0.2[a]
Average	% Viability	33.4 ± 0.3	67.4 ± 0.3[a]	47 ± 0.2[a]	44 ± 0.2[a]	64.1 ± 0.2[a]

[a] Significantly different ($p<0.05$) from the control.
[b] Measurements are mean CFU/ mL ± standard deviations of 3 replicates for all treatments.
[c] % Viability = (CFU/ml after 4 weeks storage / initial CFU/mL) × 100

Table 5.4. The pH of fermented samples after 4 weeks of storage at 4^0C [b]

Prebiotics Strains	Time	Control	Inulin	Lactulose	Raftilose	Hi-maize
ASCC 1520	0 h	6.29 ± 0.12	6.30 ± 0.08	6.24 ± 0.11	6.25 ± 0.10	6.30 ± 0.06
	4 wk	4.24 ± 0.15	4.12[a] ± 0.05	4.22 ± 0.18	4.34 ± 0.20	4.10[a] ± 0.08
ASCC 1521	0 h	6.26 ± 0.10	6.22 ± 0.15	6.23 ± 0.12	6.30 ± 0.20	6.26 ± 0.20
	4 wk	4.29 ± 0.12	4.15 ± 0.15	4.28 ± 0.30	4.28 ± 0.12	4.15[a] ± 0.05
CSCC 2607	0 h	6.31 ± 0.15	6.29 ± 0.20	6.35 ± 0.10	6.32 ± 0.14	6.38 ± 0.10
	4 wk	4.30 ± 0.21	4.12 ± 0.20	4.29 ± 0.20	4.30 ± 0.15	4.18 ± 0.18
ATCC 15820	0 h	6.22 ± 0.11	6.24 ± 0.14	6.26 ± 0.17	6.29 ± 0.11	6.32 ± 0.18
	4 wk	4.20 ± 0.09	4.10[a] ± 0.02	4.24 ± 0.22	4.29 ± 0.16	4.13 ± 0.19
ASCC 290	0 h	6.32 ±0.10	6.30 ± 0.14	6.33 ± 0.20	6.35 ± 0.14	6.33 ± 0.15
	4 wk	4.25 ± 0.16	4.13 ± 0.1	4.26 ± 0.20	4.22 ± 0.11	4.20 ± 0.2
ASCC 292	0 h	6.22 ± 0.10	6.25 ± 0.14	6.23 ± 0.10	6.30 ± 0.10	6.32 ± 0.17
	4 wk	4.27 ± 0.17	4.11 ± 0.15	4.25 ± 0.15	4.18 ± 0.15	4.21 ± 0.18
ASCC 279	0 h	6.30 ± 0.10	6.32 ± 0.20	6.33 ± 0.15	6.32 ± 0.10	6.30 ± 0.14
	4 wk	4.23 ± 0.11	4.16 ± 0.18	4.26 ±0.15	4.12 ± 0.15	4.12 ± 0.25

[a] Significantly different ($p<0.05$) from the control.
[b] Measurements are means ± standard deviations of 3 replicates for all treatments.

5.5 Conclusion

The growth, activity and viability of seven *Lactobacillus* strains in RSM were dependent on prebiotics as well as strain. Doubling time was significantly decreased with addition of prebiotics. Hence, addition of prebiotics improves the growth rates and decrease fermentation time. There was also slight improvement in survival rate with prebiotics, thus incorporating prebiotics will improve the efficiency of the product. The synbiotic functional food approach offers an additional health benefit by providing probiotics and prebiotics. With appropriate combinations of strain and prebiotic, product fermentation times could be reduced and survival of the strain during product storage increased, providing advantages to both the manufacturer and the consumer.

6.0 INVITRO INHIBITION OF *HELICOBACTER PYLORI* BY *LACTOBACILLUS CASEI* STRAINS

6.1 Introduction

Lactic acid bacteria (LAB) are widely used in the production of fermented foods, beverages and contribute to the sensory qualities and preservation of food and to the prevention of spoilage. Moreover, they are present in large numbers in the normal human and animal gastrointestinal flora (Sgouras *et al*., 2004). Health promoting benefits of consumption of LAB have been known for several years, since Metchnikoff (1908) first longevity of Bulgarian peasants to consumption of fermented milks. The term 'probiotic' was first described by Fuller (1989) as 'a live microbial feed supplement that beneficially affects the host by improving its intestinal microbial balance'. Some of the commonly known probiotics belong to the *Lactobacillus* genus. Strains of *Lactobacillus casei, Lactobacillus rhamnosus, Lactobacillus paracasei* and *Lactobacillus acidophilus* have been identified as possessing probiotic properties, and these strains have been used to treat gastrointestinal diseases. LAB have shown to exert either bacteriosatatic or bactericidal activity against several pathogens. Many probiotics are now widely incorporated in food products such as yoghurt, fermented milk, fermented or non fermented juices and marketed as freeze dried supplements.

The human gastrointestinal microflora under normal circumstances is a stable ecosystem in which the microorganisms remain relatively constant. The role of the normal microflora is still poorly understood, but two of the most important functions from the host's point view are to

maintain resistance to colonization by infections caused by pathogens and to perform certain metabolic functions. Maintenance of the intestinal ecological flora is important in preventing pathogenic bacteria. Widespread use of antibiotics has not only led to an increase in antibiotic-resistant pathogenic bacteria, but it is often associated with the disruption of the protective flora, leading to predisposition to infections. For these reasons, the control of infections through a non antibiotic approach is urgently needed and bacterial replacement therapy using natural flora is a promising alternative. Probiotic bacteria are live microorganisms which function for the well being of the host. It is accepted that these bacteria might represent effective tools for controlling overgrowth of pathogens and thereby prevent infections. Numerous in vitro and in vivo studies performed with different probiotic bacteria have shown the capabilities of these bacteria to interfere with both growth and virulence properties (Arvola *et al.*, 1999; Cocconnier *et al.*, 1998).

Helicobacter pylori is a spiral Gram-negative, microaerophilic stomach pathogen that infects over 50% of population worldwide (Sgouras *et al.*, 2004). Marshall and Warren (1984) first isolated it in 1984 in gastric biopsy samples from patients suffering from gastritis and peptic ulcers. Following their discovery, investigators all over the world rapidly confirmed the presence of these organisms in the gastric mucus. It is now evident that once acquired, *H. pylori* persists, usually for life, unless eradicated by antimicrobial therapy (Dunn *et al*., 1997). It is a specialised pathogen that has a unique combination of virulence factors, an incomplete citric acid cycle, a simple respiratory chain with only a single terminal oxidase and few regulatory systems (Kelly, 2001). In antral and duodenal biopsy specimens, *H. pylori* has been shown to attach to epithelial cells and occasionally penetrate the cells (Coconnier *et al*., 1998). Chronic infection with this bacterium has been identified as the major etiological factor in gastritis, gastric ulcers, gastric atrophy, and gastric carcinoma. As a result, in 1994, the International Agency for Research on

Cancer (IARC, Lyon, France), classified *H. pylori* infection as a carcinogenic agent class I (Van de Bovenkamp *et al.,* 2003).

Several authors have reported antagonistic activity of *Lactobacillus* against *H. pylori.* Coconnier *et al.* (1998) studied the human *L. acidophilus* strain LB, which secretes an antimicrobial substance and found that the spent culture supernatant of the strain dramatically decreased the viability of *H. pylori* in vitro. The adhesion of *H. pylori* to HT29-MTX cells also decreased as did their viability in the presence of the supernatant. Inhibition of stomach colonization by *H. felis* in conventional mice was also observed by the authors. Sgouras *et al.* (2004) reported in vitro activity against *H. pylori* in the presence of viable *L. casei* shirota cells. Antimicrobial activity of probiotic bacteria has been attributed to the antimicrobial substances released by the organisms. These include several metabolites, organic acids and bacteriocins. Several studies also proposed that lactic acid production by these organisms is responsible for inhibition of *H. pylori* (Midolo et *al.,* 1995; Bhatia *et al.,* 1989). There is a growing interest in finding safer, 'side-effect free' ways of treating infections, and probiotic bacteria can play a very important role.

In this study the antimicrobial effects of *Lactobacillus* were tested against 2 patient strains of *H. pylori* and a type strain ATCC 11637 using well diffusion assays, and co-culturing of *Lactobacillus* and *H. pylori*.

6.2 Material and methods

The *H. pylori* was grown in horse blood agar at 35^0C for 72 h at microaerophilic condition. Subculturing was performed in the same agar for 72 h at 35^0 C and the organisms were preserved by keeping at -80^0C in horse blood broth.

6.2.1 Bacterial strain

Twenty two different strains of *Lactobacillus casei* group were obtained from Australian Starter Culture Research Centre (ASCRC, 180 Princes Hwy, Werribee, Victoria, Australia). The organisms were activated by growing 3 times in MRS broth at 37^0C for 24 h. The *H. pylori* used in this study included one type strain ATCC *H. pylori* 11637, two patient's isolates *H. pylori* Fiona 21174 and *H. pylori* U 3119288 obtained from Monash Medical Centre (Clayton, Victoria, Australia). All three were grown in BHI broth under anaerobic condition for 72 h at 35^0C.

6.2.2 Growth inhibition of H. pylori with Lactobacillus as measured by inhibition assays

All twenty two strains of *Lactobacillus casei* were grown at 37^0C for 24 h using 1% inoculum for three times. To obtain supernatant, 200 mL aliquots of all twenty two strains were centrifuged at 3838×g for 15 min at 4^0C. The supernatant was divided into two equal portions in test tubes. The pH of the first portion was adjusted to 7.0 using 2 N NaOH. The pH was measured at 20-22^0C using a pH meter (Model 8471; Hanna Instruments Pty Ltd., Singapore) after calibrating with fresh pH 7.0 and 4.0 buffers. The pH of the second portion was recorded and was left unadjusted. Both supernatants were then filtered using 0.45 µm millipore filters (Millipore Corp., Bedford, MA, USA). A thick lawn of each strain of *H. pylori* was prepared by spreading 0.5 mL culture on to BHI agar plates. Each plate was divided into 4 quadrants and wells were cut in the agar using a sterile 6 mm borer. The bottom of each well was sealed with 0.9% sterile agar followed by addition of 100 µL of supernatant of each *Lactobacillus* strains. All inhibition assays

were performed anaerobically in triplicate for each pathogenic organism, for both adjusted and unadjusted supernatant. The supernatant was allowed to diffuse for 2 h into agar and incubated at 35^0C for 72 h. The inhibition zones were measured.

6.2.3 Effect of growing H. pylori with supernatants obtained from Lactobacillus strains

The cultures were activated by 3 consecutive transfers and supernatants were obtained as discussed before. The supernatant was divided into two portions and one portion was left unadjusted, while the other portion was adjusted to pH 7.0 using 5 N NaOH. One millilitre of the neutralised or unnaturalised supernatant and 1 mL of *H. pylori* type strain 11637 and two patients isolates Fiona 21174 and U 3119288 were added to 10 mL BHI broth in McCartney bottles, followed by incubation at 35^0C for 24 h. The control contained 10 mL of BHI broth, 1 mL distilled water and 1 mL of each pathogenic microorganisms. Growth inhibition of *H. pylori* was monitored by measuring optical density using Novaspec II spectrophotometer (Pharmacia, Biotech, LKB Biochrom, UK) at 620 nm for every 12 h for 72 h.

6.2.4 Effect of growing H. pylori and Lactobacillus in the presence of supernatant obtained from Lactobacillus

The effects of supernatant obtained from each *Lactobacillus* on the growth of *H. pylori* were assessed by adding separately 1 mL each of the seven *Lactobacillus* supernatants to 1 mL aliquot of *H. pylori* in 10 mL BHI broth, followed by incubation at 35^0C for 72 h. The control contained 1 mL of distilled water instead of the supernatant. Aliquots were withdrawn at 0, 24, 48 and 72 h to measure optical density and BHI agar was used to enumerate *H. pylori*. The plates were incubated under anaerobic conditions 35^0C for 72 h.

6.2.5 Effect of co-culturing Lactobacillus and selected pathogens in BHI broth

The effect of co-culturing of all seven *Lactobacillus* strains with all 3 *H. pylori* were assessed individually by mixing 1 mL of each of them to 10 mL BHI broth and the co-cultures were grown

anaerobically for 72 h. The control contained 1 mL of sterile distilled water instead of *Lactobacillus* cells. One millilitre aliquot was obtained at 12 h intervals for measurement of pH, optical density and enumeration for both groups of bacteria.

Enumeration of *Lactobacillus* was performed by pour plating using MRS agar with 0.1% vancomycin. The plates were incubated aerobically at 37^0C for 72 h. BHI agar was used to enumerate *H. pylori* and plates were incubated under anaerobic conditions at 35^0C for 72 h.

6.2.6 Activity determination of Lactobacillus strains

The activity of each *Lactobacillus* strain was determined by measuring end product of fermentation (lactic and acetic acids) using high-performance liquid chromatography (HPLC; Varian Australian Ltd, Mulgrave, Australia). Each *Lactobacillus* strains was grown in MRS broth as described previously. HPLC analysis was performed using the method described by Dubey and Mistry (1996). One hundred microliters of 15.8 M HNO_3 and 14.9 mL of 9 mM H_2SO_4 were added to 1.5 mL of sample and centrifuged at 4000 × g for 10 min using a bench top centrifuge (Sorvall RT7, Newtown, CT, USA). Two millilitre aliquots were stored at -20^0C until analysed. An Aminex HPX-87H column with disposable cartridges at 65^0C was used for organic acid analysis. The degassed mobile phase of 9 mM $H_2 SO_4$, filtered through a 0.45 µm membrane filter (Millipore), was used at a flow rate of 0.3 mL/min. The wavelength of detection was optimised at 220 nm for the organic acids being quantified. Standard solutions of organic acids (lactic and acetic acids; Sigma Chemical Co., St Louis, MO, USA) were prepared in mobile phase to establish elution times and calibration curves. The retention times and standard co-efficient for acetic and lactic acids were 16.2 and 25.7 min and 0.9992 and 0.99946, respectively.

6.3 Results and discussion

6.3.1 Growth inhibition of H. pylori with Lactobacillus as measured by inhibition assays

The growth inhibition of three *H. pylori* strains with 22 different strains of *Lactobacillus casei* group is shown in Table 6.1. In general, the zones of inhibitions were greater with the addition of supernatants of three strains of *L. rhamnosus* ASCC 1520, 1521 and 2607, two strains of *L. paracasei* ASCC 292 and 279, one strain each of *L. casei* ASCC 290 and *L. zeae* ATCC 15820 respectively. These strains were used for further study against *H. pylori*. The antimicrobial effects of *Lactobacillus* are attributed generally to the decrease in pH resulting from the production of lactic and acetic acids. These results are comparable to those of Desai *et al.* (2004). The final pH of the unadjusted supernatant decreased from 6.4 at 0 H to pH 3.8 to 4.02 at 24 h depending on the strain (Table 6.1). We selected as earlier described seven robust *Lactobacillus* strains for further study.

6.3.2 Effect of growing H. pylori with Lactobacillus supernatants

The suppression of growth of 3 different strains of *H. pylori* with seven *L. casei* supernatants as measured by decrease in OD is presented in Table 6.2a and b. In general, the Three pathogenic bacteria were greatly inhibited when grown in the presence of supernatant of *Lactobacillus* strains over 48 h as indicated by the decrease in OD. Addition of unadjusted supernatant of all seven strains of *Lactobacillus* showed a stronger inhibition against pathogenic bacteria. While pH adjusted supernatants of *L. casei* 290, 292, 1521 and 1520 showed significant inhibition (10-34%) against pathogenic bacteria. The results are consistent with greater zone of inhibition with all above shown *Lactobacillus* strains. The pH of the supernatant was 3.90 for *L. casei* (290), 2.89 for *L. casei* (292), 3.92 for *L. casei* (1521) and 3.87 for *L. casei* (1520).

6.3.3 Effect of growing H. pylori and Lactobacillus in the presence of Lactobacillus supernatant

The effect of growing *H. pylori* with supernatant obtained from *H. pylori* with supernatant obtained from *Lactobacillus* strains (290, 292, 1520, 15820, 279, 2607 and 521) are mixed together as measured by OD and bacterial counts are shown in Table 6.3. In general, there was a decrease in OD at 72 h. A similar trend was observed with bacterial counts. The inhibition of *H. pylori* was significant ($p < 0.05$) when supernatant was not neutralised. When supernatant was neutralised, the OD readings were also significant ($p < 0.05$) for *Lactobacillus* (290, 292, 1521 and 1520) (Table 6.3).

6.3.4 Effect of co-culturing Lactobacillus and selected pathogens in BHI broth

The effect of co-culturing *Lactobacillus* spp (1521, 1520, 290 and 292) and pathogenic bacteria *H. pylori* is shown in Table 6.4 respectively. When pathogens were co-cultured with *Lactobacillus* strains, there was a decrease in cell density. Similarly, the pH decreased by 1.42 to 1.91 log units when *Lactobacillus* was added with the pathogenic bacteria. There was a substantial decrease of all of the pathogenic bacteria when above mentioned *Lactobacillus* added. This decrease ranged from 32.7 – 94.7% with a decrease in pH between 1.42- 1.91.

6.3.5 Activity determination of Lactobacillus

Lactobacillus group of organisms include facultative hetero-fermentative strains that produce lactic and acetic acids as the end products of glycolysis (Limsowtin *et al.*, 2003). This is an important characteristic of *Lactobacillus*. As per shown in Table 6.5 the highest level of acetic acid (117.6 ± 4.8) produced by *L. casei* 15820 while the lowest level (31.1 ± 0.5) was produced

by *L. casei* 2607. The highest level of lactic acid (1464 ± 30) was produced by *L. casei* 2607 while lowest level (1056 ± 3.1) was produced by *L. casei* 292.

6.4 Tables

Table 6.1: Inhibition of *H. pylori* with 22 strains of *L. casei* supernatant

Organisms List	pH	*H. pylori*(HP 11637)	Patient isolate Fiona 21174	Patient isolate U 3119288
		Zone of inhibition (mm)[1,2,3]		
ASCC 290	3.8	13.08 ± 0.9	13.22 ± 1.1	13.4 ± 1.0
CSCC 5203	3.85	12.02 ± 0.8	12.11 ± 1.1	11.89 ± 0.7
ATCC 15820	4.01	11.85 ± 0.4	11.58 ± 0.5	11.62 ± 0.8
ASCC 292	3.89	12.33 ± 0.8	12.42 ± 0.9	12.75 ± 0.5
ASCC 1521	3.92	13.2 ± 0.6	12.9 ± 0.9	12.55 ± 0.4
ASCC 295	3.96	10.1 ± 0.2	10.3 ± 0.6	10 .0 ± 0.8
ASCC 526	4.03	9.8 ± 0.8	9.7 ± 0.4	9.2 ± 0.2
ASCC 1180	3.86	9.0 ± 0.2	9.1 ± 0.1	9.3 ±0.6
ASCC 279	3.95	10.22 ± 0.8	11.44 ±1.3	11.25 ± 1.1
ASCC192	3.88	11.22 ± 0.4	10.92 ± 0.6	8.5 ± 0.2
ASCC 277	3.9	7.75 ± 1.0	7.67 ± 0.9	7.50 ± 0.6
ASCC 1002	3.87	8.17 ± 1.3	8.11 ± 0.6	8.33 ± 1.4
ASCC 1181	4.02	9.00 ± 1.6	9.1 ± 2.0	9.8 ± 0.8
ASCC 1519	3.81	10.0 ± 1.8	7.11 ± 0.6	7.92 ± 0.5
ASCC 1520	3.87	12.01 ± 0.5	10.6 ±0.8	12.10 ± 0.2
ASCC 1522	3.9	10.8 ± 0.2	10.25 ± 0.2	10.15 ±1.1
ASCC 1523	3.94	7.99 ± 1.1	7.82 ± 1.4	7.67 ± 0.9
CSCC 2603	3.81	8.00 ± 0.9	8.17 ± 0.8	7.95 ± 1.1
CSCC 2605	3.89	7.02 ± 1.05	7.59 ± 1.6	7.83 ± 1.2
CSCC 2607	3.88	12.25 ± 0.2	12.01 ± 0.4	12.69 ± 0.8
CSCC 2627	3.89	10.31 ± 0.9	10.83 ± 0.41	9.00 ± 1.0
CSCC 5376	3.98	8.33 ± 1.4	8.67 ± 0.5	8.12 ± 0.9

[1] Data are the means and standard deviations of two independent experiments (n=6).
[2] Zone of inhibition includes a 6mm bore diameter.
[3] Incubation condition were: (CO_2 / 72 h/ 35^0C).

Table 6.2a: OD at 620 nm [2] Growth of *Lactobacillus* strains with Supernatant obtained from *H. pylori* [5]

	control	**290**		**15820**		**292**		**1521**	
		Adj[3]	**Undaj[4]**	**Adj[3]**	**Undaj[4]**	**Adj[3]**	**Undaj[4]**	**Adj[3]**	**Undaj[4]**
H. pylori (HP 11637)	0.88	0.72	0.62	0.82	0.42	0.56	0.5	0.61	0.5
H. pylori (21174)	0.9	0.78	0.52	0.88	0.46	0.6	0.39	0.58	0.45
H. pylori (3119288)	0.98	0.81	0.48	0.92	0.59	0.64	0.41	0.66	0.36

279		**1520**		**2607**	
Adj[3]	**Undaj[4]**	**Adj[3]**	**Undaj[4]**	**Adj[3]**	**Undaj[4]**
0.85	0.44	0.78	0.41	0.89	0.45
0.8	0.59	0.7	0.43	0.87	0.55
0.91	0.63	0.62	0.36	0.91	0.68

[1]Percentage difference in optical density between control and pathogenic bacteria with supernatant obtained from Lactobacilli.

[2]optical density measured at a wavelength of 620nm.

[3]Supernatant was adjusted at pH 7.

[4]Supernatant left unadjusted.

[5]Data are the means of two independent experiments (n=6).

Table 6.2b: Suppression of growth of pathogenic bacteria in the presence of supernatant obtained from lactobacilli [5]

	Lactobacilli strains							
	290 [1]		**15820 [1]**		**292 [1]**		**1521 [1]**	
	Adj[3]	**Undaj[4]**	**Adj[3]**	**Undaj[4]**	**Adj[3]**	**Undaj[4]**	**Adj[3]**	**Undaj[4]**
H. pylori (HP 11637)[1]	-28.0%	-38.0%	-18.0%	-58.0%	-44.0%	-50.0%	-39.0%	-50.0%
H. pylori (21174)[1]	-22.0%	-48.0%	-12.0%	-54.0%	-40.0%	-61.0%	-42.0%	-55.0%
H. pylori (3119288)[1]	-19.0%	-52.0%	-8.0%	-41.0%	-36.0%	-59.0%	-34.0%	-64.0%

279 [1]		**1520 [1]**		**2607 [1]**	
Adj[3]	**Undaj[4]**	**Adj[3]**	**Undaj[4]**	**Adj[3]**	**Undaj[4]**
-15.0%	-56.0%	-22.0%	-59.0%	-11.0%	-55.0%
-20.0%	-41.0%	-30.0%	-57.0%	-13.0%	-45.0%
-9.0%	-37.0%	-38.0%	-64.0%	-9.0%	-32.0%

[1]Percentage difference in optical density between control and pathogenic bacteria with supernatant obtained from Lactobacilli.
[2]optical density measured at a wavelength of 620nm.
[3]Supernatant was adjusted at pH 7.
[4]Supernatant left unadjusted.
[5]Data are the means of two independent experiments (n=6).

Table 6.3: Inhibition of *H. pylori* with supernatant obtained from Lactobacilli as measured by OD and bacterial counts[6]

				Lactobacilli strains						
				290		**292**		**1521**		
		Bacterial counts	**Control**	**Adj[3]**	**Undaj[4]**	**Adj[3]**	**Undaj[4]**	**Adj[3]**	**Undaj[4]**
H. pylori	(HP 11637)[5]	OD at 72h[1]	0.9	0.62	0.51	0.56	0.5	0.61	0.48
		CFU at 72h[2]	1.11×10^{10}	8.21×10^{8}	5.95×10^{6}	5.21×10^{8}	3.21×10^{6}	1.21×10^{8}	2.02×10^{6}
H. pylori	(21174)[5]	OD at 72h[1]	0.92	0.72	0.41	0.6	0.39	0.58	0.31
		CFU at 72h[2]	1.21×10^{10}	1.35×10^{8}	8.65×10^{6}	4.25×10^{8}	3.68×10^{6}	7.42×10^{8}	2.58×10^{6}
H. pylori	(3119288)[5]	OD at 72h[1]	1.12	0.82	0.62	0.94	0.61	0.88	0.51
		CFU at 72h[2]	1.80×10^{10}	5.55×10^{8}	7.23×10^{6}	6.12×10^{8}	3.91×10^{6}	5.551×10^{8}	1.90×10^{6}

1520		**15820**		**279**		**2607**	
Adj[3]	**Undaj[4]**	**Adj[3]**	**Undaj[4]**	**Adj[3]**	**Undaj[4]**	**Adj[3]**	**Undaj[4]**
0.78	0.41	0.86	0.32	0.71	0.34	0.78	0.41
6.21×10^{9}	1.55×10^{7}	4.21×10^{9}	1.02×10^{7}	5.12×10^{8}	1.45×10^{7}	422×10^{9}	9.92×10^{7}
0.7	0.42	0.65	0.049	0.77	0.38	0.7	0.42
8.54×10^{9}	1.32×10^{7}	3.35×10^{9}	4.28×10^{7}	6.68×10^{8}	1.25×10^{7}	5.85×10^{9}	1.02×10^{7}
0.83	0.56	0.71	0.39	0.74	0.38	0.51	0.27
9.51×10^{9}	1.22×10^{7}	5.22×10^{9}	3.64×10^{7}	8.85×10^{8}	1.14×10^{7}	6.12×10^{9}	8.89×10^{7}

[1]Optical density measured at a wavelength of 620 nm. [2]CFU = colony forming units per ml.
[3]Supernatant was adjusted at pH 7. [4]Supernatant left unadjusted.
[5]*H.pylori* was grown on BHI agar (Co_2 / 35^0C / 72h). [6]Data are the means of two independent experiments (n=6).

Table 6.4: Effect of co- culturing with *H. pylori* and four strains of Lactobacilli after 72 h[1]

	Control			Co-culture[3]					
	OD_{620}2	CFU[4]	pH	OD_{620}	% OD difference	CFU	%CFU difference	pH	pH difference
L. casei 1521									
H. pylori (HP 11637)[1]	1.44	1.32×10^9	6.12	1.13	-21%	3.21×10^8	-76%	4.44	-1.68
H. pylori (21174)[1]	1.01	1.32×10^9	6.01	0.91	-9%	6.61×10^8	-50%	4.52	-1.49
H. pylori (3119288)[1]	1.29	9.95×10^9	6.22	.95	-26%	5.58×10^8	-94%	4.62	-1.6
L. casei 1520									
H. pylori (HP 11637)[1]	1.44	1.32×10^9	6.12	1.59	11%	4.25×10^8	-68%	4.58	-1.54
H. pylori (21174)[1]	1.01	1.32×10^9	6.01	1.01	0%	8.88×10^8	-33%	4.42	-1.59
H. pylori (3119288)[1]	1.29	9.95×10^9	6.22	1.12	-11%	5.25×10^8	-95%	4.80	-1.42
L. casei 290									
H. pylori (HP 11637)[1]	1.44	1.32×10^9	6.12	1.69	18%	6.38×10^8	-52%	4.21	-1.91
H. pylori (21174)[1]	1.01	1.32×10^9	6.01	0.89	-11%	7.75×10^8	-41%	4.50	-1.51
H. pylori (3119288)[1]	1.29	9.95×10^9	6.22	1.29	0%	9.18×10^8	-91%	4.71	-1.51
L. casei 292									
H. pylori (HP 11637)[1]	1.44	1.32×10^9	6.12	1.55	8%	4.25×10^8	-68%	4.32	-1.8
H. pylori (21174)[1]	1.01	1.32×10^9	6.01	0.92	-8%	5.28×10^8	-60%	4.49	-1.52
H. pylori (3119288)[1]	1.29	9.95×10^9	6.22	1.30	1%	6.28×10^8	-94%	4.63	-1.59

[1] Data are the means of 2 independent experiments (n=6). [2] Optical density of pathogenic bacteria.

[3] Co-culture of pathogenic bateria and four *L. casei* strains.

[4] Colony forming units of different *H. pylori* strains.

Table 6.5: Acetic and lactic acid production by seven Lactobacilli strains

Lactobacilli strains	Acetic acid(mg/L)[1]	Lactic acid (mg/L)[1]
*L.casei*290	76.4 ± 2.1	1439 ± 73
L.casei 15820	117.6 ± 4.8	1350 ± 26
*L. casei*292	105.9 ± 3.1	1056 ± 31
*L. casei*1521	53.4 ± 1.0	1438 ± 73
*L. casei*279	97.6 ± 2.1	1245 ± 21
*L.casei*1520	51.7 ± 1.7	1457 ± 5
*L. casei*2607	31.1 ± 0.5	1464 ± 30

[1] Data are the means and standard deviations of three independent experiments (n=6)

6.5 Conclusion

Lactobacillus spp ASCC290, ASCC292, ASCC1520, ASCC 279, ASCC 1521, CSCC 2607, & ATCC 15820 were able to inhibit pathogenic and putrefactive micro-organisms as measured by inhibition assays and during growth with supernatants obtained from *Lactobacillus* spp. This inhibition of pathogenic and putrefactive bacteria may be due to the lowering pH by the *Lactobacillus* strains. When the pH of the supernatant was adjusted to natural, the inhibition of growth and zones were due to unknown antibacterial substances produced by probiotic bacteria. These strains gave promising results against peptic ulcer causing *Helicobacter pylori.* Further in vivo study is needed to confirm antimicrobial properties of *Lactobacillus* strains.

8.0 FUTURE RESEARCH DIRECTIONS

In developed world, lactic acid bacteria are mainly associated with fermented dairy products such as cheese, buttermilk and yoghurt. The use of these bacteria has been extensive in this century not only as a starter culture they are also associated with beneficial health effects. Today, an increasing number of health food as so-called functional food as well as pharmaceutical preparations are promoted with health claims based on the characteristics of certain strains of lactic acid bacteria. Knowledge of probiotic bacteria and used with different prebiotics increases globally, the demand for products containing probiotic bacteria have increased significantly. There has been increasing range of probiotic prtoducts produced of dairy origin such as yoghurt, fermented milks and other dairy products while consumption of tablets or capsule containing freeze-dried probiotic organisms have become increasingly popular due to their higher bacterial concentrations.

Further research into the fermentation of different types of prebiotics by probiotic bacteria is critical to establish which products produce the greatest synbiotic response. Research into the mechanisms as to why certain prebiotics provide a greater beneficial effect than others to particular probiotic strains would improve knowledge of the reactions that take inside the gastrointestinal tract.

While knowledge of some probiotic strains for their acid, bile tolerance and bile deconjugation ability has been ascertained as well as antimicrobial ability and anti-carcinogenic properties, further work is still needed on improving viability of probiotic strains in commercial products and determining the concentration of probiotic bacteria for daily consumption in order to achieve health benefits.

The inhibitory ability of probiotic bacteria especially lactobacilli towards *Helicobacter pylori* has important clinical implications. Due to the exponential increase in resistance to antibiotics by many bacterial species, research on probiotics is imperative to successfully demonstrate effectiveness in reduction and elimination of these strains from human stomach.

Our work has shown that several *Lactobacillus* strains are able to successfully inhibit *Helicobacter pylori* in vitro. These strains need to be further assessed and critically evaluated by clinical research on patients colonised and infected with *Helicobacter pylori*. It may be useful to study which probiotic strain(s) combined with prebiotics are most successful in eliminating *H. pylori in vivo*. Without the use of antibiotics, which cause disturbance in the balance of the gastrointestinal microflora.

7.0 OVERALL CONCLUSION

7.1 Discrimination of dairy isolates of the *Lactobacillus casei* group

In this study, we have confirmed that a set of simple PCR tests based on 16S rRNA gene sequences, coupled with PFGE, can be used to distinguish *Lactobacillus* strains of the *L. casei* group. Pulsed field gel electrophoresis discriminated 19 different fragment patterns from the 22 strains studied. Carbohydrate fermentation tests classified lactobacilli at genus level but the test were not reliable at species level. Using three different methods, we obtained consistent PCR identification of *L. rhamnosus.* We distinguished *L. paracasei* and *L. casei* by using primers targeting 16S rRNA genes. We also distinguished *L. zeae* by using primers targeting 16-23S rRNA spacer regions. Partial 16S rRNA gene sequences of each species confirmed specificity of PCR results. These results are consistent with the current taxonomy of the *L .casei* group.

The usefulness of sugar fermentation tests is limited to identification of *L. paracasei* and *L. rhamnosus*. The results indicate that *L. casei* and *L. zeae* are not common in dairy environments and that (at least within the collection studied) the prevalence of *L. rhamnosus* has been historically underestimated. Reclassification of strains described in the literature and in industry practice is recommended

7.2 Investigation of tolerance of *Lactobacillus* strains to the presence of acid, bile salts and deconjugated bile salts.

Twenty two different *Lactobacillus* strains were screened under harsh conditions of acid and all showed good to excellent tolerance against acid. Similarly all twenty two different *Lactobacillus* strains showed moderate to good tolerance against bile salts.

Overall following seven *Lactobacillus* strains, ASCC 1520, ASCC 1521, ASCC 279, ASCC 290, ASCC292, ATCC 15820 & CSCC 2607 have shown excellent acid and bile tolerance. So we selected seven robust *Lactobacillus* strains for their deconjugation activity. They were screened for their deconjugation ability with sodium glycocholate and sodium taurocholate. Results were determined by measuring quantity of cholic acid. All strains released between 1.16 to 4.70 mM. All seven strains were able to deconjugate both sodium glycocholate and sodium taurocholate at different level. ASCC 1521 was highest in deconjugation activity followed by ATCC 15820 and ASCC 1520. ASCC 1520 showed lowest deconjugation activity. All seven strains showed lower deconjugation of sodium taurocholate compared to sodium glycocholate. Mixture of sodium glycocholate and sodium taurocholate at similar molar ratio in human bile, all seven strains showed consistent cholic acid secretion. ASCC 1521 also showed highest cholic acid liberation while ASCC 1520 showed lowest deconjugation ability.

7.3 Viability and proteolytic activity of freeze dried *Lactobacillus* strains at various temperatures during storage

Long term storage at -18^{0}C, 4^{0}C and 20^{0}C of freeze dried seven *Lactobacillus* strains was examined. All seven *Lactobacillus* strains provided maximum viability and proteolytic activity at -18^{0}C. The bacterial counts reduced at refrigerated temperatures (4^{0}C), whereas storage temperature of 20^{0}C reduced the viable count significantly. There was no significant cont was noted after two months of storage. This study has established that a temperature maintained at -18^{0}C was ideal for the long term storage for *Lactobacillus* strains. All *Lactobacillus* strains stored at -18^{0}C shown higher proteolytic activity up to 12 mo of storage. These results gave promising indication of future commercial use of *Lactobacillus* strains either in pharmaceutical or cultured milk products

7.4 Survival and activity of *Lactobacillus casei* spp. in skim milk containing prebiotics

The growth, activity and viability of seven strains of *Lactobacillus* in skim milk were dependent on the prebiotic present as well as the strain of *Lactobacillus.* Doubling time was significantly decreased with the addition of prebiotics lactulose, raftilose and inulin although Hi maize^{TM} gave higher doubling time than control.

Fermentation of the four prebiotics with seven strains of *Lactobacillus* was assessed. The highest level of lactic acid was produced by ATCC 15820 with Hi-maize and lowest by ASCC 292 with raftilose. The highest level of acetic acid was produced by ATCC 15820 with lactulose while the lowest by CSCC 2607 with Hi-maize. The addition of prebiotics viability was improved. The highest viability of 83% was recorded for CSCC 2607 with inulin. Overall raftilose and lactulose were the least effective in maintaining viability, with average viabilities of 44% and 47%. The lowest viability was recorded by ASCC 1521 with an average viability of 35%. The pH of fermented samples after 4 weeks of storage ranged from 4.34 (ASCC 1520 with raftilose) to 4.10 (ATCC 15820with inulin.

7.5 In vitro inhibition of *Helicobacter pylori* by *Lactobacillus casei* strains

Twenty two different strains of *Lactobacillus casei* group were assessed against three strains of *Helicobacter pylori*. In general the zone of inhibitions were greater with the addition of supernatants of *Lactobacillus* spp ASCC290, ASCC292, ASCC1520, ASCC 279, ASCC 1521, CSCC 2607, & ATCC 15820 This inhibition of pathogenic and putrefactive bacteria may be due to the lowering pH by the *Lactobacillus* strains. When the pH of the supernatant was adjusted to natural against pathogenic bacteria the results were consistent with greater zone of inhibition with all above seven strains.

The effect of growing *H. pylori* with supernatant obtained from *H. pylori* with supernatant obtained from *Lactobacillus* strains (290, 292, 1520, 15820, 279, 2607 and 521) are mixed together as measured by OD shown similar decrease in bacterial count at 72 h. The effect of co-culturing was also examined with *Lactobacillus* spp. (1521, 1520, 290 and 292) showed decrease in cell density. Similarly, the pH decreased by 1.42 to 1.91 log units. There was substantial decrease of all of the pathogenic bacteria when above mentioned *Lactobacillus* strains were added. These strains gave promising results against peptic ulcer causing *Helicobacter pylori.* Further in vivo study is needed to confirm antimicrobial properties of *Lactobacillus* strains.

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