INHIBITORY SUBSTANCES PRODUCED BY PROBIOTIC BACTERIA FOR CONTROL OF FOOD-BORNE PATHOGENIC AND SPOILAGE MICROORGANISMS IN DIPS

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by

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DEDICATION

I dedicate this thesis to my parents who have always believed in me and generously supported me in all my efforts.

ABSTRACT

The success in using a food product as a delivery vehicle for probiotics depends on its ability to maintain required level of viable cells (at least 10^7 cfu g^{-1}) and to suppress the growth of spoilage and pathogenic organism. Cheese-based dips could delivery probiotic bacteria owing to its stable pH, buffering capacity of ingredients and the presence of prebiotics. The anti-microbial properties of probiotics can also be employed for controlling the spoilage organisms such as yeast and mould.

The work described in this thesis focused on the survival of probiotics and their anti-microbial effects in dips. Effective selective enumeration methods were first identified for specific probiotic cultures to enumerate their numbers, the ideal conditions in which the organisms survive better were evaluated and the mechanism by which the probiotic organisms antagonise pathogenic and spoilage organisms were then elucidated. The cultures of *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Streptococcus thermophilus*, *Lactobacillus casei*, *Lactobacillus rhamnosus*, *Lactobacillus acidophilus*, *Bifidobacterium* spp. and propionibacteria were tested in selected bacteriological media to evaluate their suitability as selective media. Nineteen bacteriological media were evaluated at different incubation conditions, including *Streptococcus thermophilus* (ST) agar, pH-modified MRS agar, MRS-vancomycine agar (MRS-V agar), MRS-bile agar, MRS-NaCl agar, MRS-lithium chloride agar, MRS-NNLP agar, RCA agar, sugar-based (such as maltose, galactose, sorbitol, manitol, esculin) agar media, sodium lactate agar (NaLa), arabinose agar, raffinose agar, xylose agar and LC agar. Aerobic and anaerobic incubations were carried out at temperatures of 27°C, 30°C, 37°C, 43°C and 45°C for the duration of 24h, 72h and 7-9 days. ST agar and aerobic incubation at 37°C for 24h were suitable for *S. thermophilus*. *L. delbrueckii* ssp *bulgaricus* can be enumerated in MRS agar (pH 4.58 or pH 5.20) and anaerobic incubation at 45°C for 72h. MRS- V agar and anaerobic incubation at 43°C for 72h was suitable to enumerate *L. rhamnosus*. Anaerobic incubation in MRS-V agar at 37°C for 72h was selective to enumerate *L. casei*. It is recommended that subtraction method should be implemented when *L. rhamnosus* is present in the product. To do this, the count of *L. rhamnosus* recorded on MRS-V agar at 43°C for 72h under anaerobic incubation should be subtracted from the total count of *L. casei*. and *L. rhamnosus* recorded on MRS-V agar 37°C for 72h under anaerobic incubation.

L. acidophilus can be enumerated on MRS-agar at 43°C for 72h under anaerobic incubation or in MRS-maltose at 43°C under anaerobic incubation for 72h or on MRS-sorbitol agar 37°C for 72h under anaerobic incubation. Bifidobacteria can be enumerated on MRS-NNLP (nalidixic acid, neomycine sulfate, lithium chloride and paramomycine sulfate) agar. Propionibacteria can be enumerated on sodium lactate (NaLa) agar. Most suitable method for counting propionibacteria in the presence of lactic acid bacteria in a product was the subtraction method. In this method, day3 count on NaLa agar under anaerobic incubation at 30°C of lactic acid bacteria was subtracted from the day 7 count (total count) of lactic acid bacteria and propionibacteria under the same incubation conditions, to obtain the propionibacteria count.

Selected probiotic bacteria were then evaluated for survival in dips. Effects of organic acids, oils and gums on the survival of probiotics in cheese-based dips were also studied. The population of *L. acidophilus* and *B. animalis* was reduced in the dips by 1 log and 2 logs, respectively. However, when the inoculation level of these bacteria was increased to 8 logs, they maintained a population of more than 6 logs over the shelf life. *L. paracasei* subsp. *paracasei* and *L. rhamnosus* remained at the inoculated level or increased slightly during the storage. Although, the population of *P. freudenreichii* subsp. *shermanii* declined by 3 logs from the inoculated level during the first couple of weeks, their numbers increased rapidly thereafter above the inoculated level.

Spot-on-lawn assay was used to identify the bacterial species/strains and their ideal ratio/s that do not antagonize each other. Between- and within- species antagonism was observed among *L. acidophilus, B. animalis, L. paracasei* subsp. *paracasei, L. rhamnosus* and *P. freudenreichii* subsp. *shermanii. L. paracasei* subsp. *paracasei* and *L. rhamnosus* species showed the greatest antagonism to all indicator bacterial species tested. *B. animalis* and *P. freudenreichii* subsp. *shermanii* did not inhibit any of the bacterial species or strains tested, except that *B. animalis* showed moderate inhibition to *L. rhamnosus* strain LC705. *L. acidophilus* strain LAC1, *B. animalis* strain BB12 and *L. paracasei* subsp. *paracasei* strains either LC01or LBC81 were found to be the best combination (ABC) for a probiotic consortium that can survive best in a food application. When used in combination, *L. paracasei* subsp. *paracasei* and *L. rhamnosus* should be included at a relatively

lower ratio (at least 1log less) to *L. acidophilus*, *B. animalis* and *P. freudenreichii* subsp. *shermanii*.

Well-diffusion-assay was used to determine the nature of the inhibitory substance/s produced by the producing bacteria against other bacteria. Due to their relative ability to tolerate aerobic condition, *L. casei*, *L. paracasei* and *L. rhamnosus* were able to establish and show some inhibitory action against *E. coli, S. typhimurium*, and *S. aureus*. Under anaerobic conditions, all producer organisms have produced considerable inhibition zones against all pathogenic bacteria. Among all the probiotic and spoilage bacterial interactions, the spore-formers were inhibited by the probiotics to a greater extent than the non-spore formers. Also, the Gram positive bacteria were inhibited more than the Gram negative bacteria. HPLC analysis of the supernatant indicated the presence of organic acids (acetic, lactic, formic, propionic butyric, benzoic and phenyl lactic acids) in varying quantities, suggesting possible involvement of these organic acids in the inhibition.

Out of the *L. acidophillus* strains, LAC1 grew faster and inhibited both Gram positive and Gram negative bacteria better than LA5. The strain BB12 was better for both types of pathogenic bacteria among the *B. animalis* strains tested. The *L. casei* strain YLC was the best among the three, *L. casei* and *L. paracasei* subsp. *paracasei* strains tested followed by *L. paracasei* subsp. *paracasei* strain LCS1. Out of the *L. rhamnosus* strains, LR1524 and GG were found to be better for both types of pathogenic and spoilage bacteria.

The inhibitory effects of all probiotic bacteria and strains were the weakest against *E. coli*. *S. aureus* was inhibited to a greater extent by *B. animalis* and *L. rhamnosus* than the other probiotic bacteria. The level of reduction in the population of pathogenic bacteria by probiotic bacteria was greatest on *B. cereus* (by 3.6 log units), followed by *S. typhimurium* (by 3.2 log units), *S. aureus* (by 2.6 log units) and *E. coli* (by 1.6 log units). *P. freudenreichii* subsp. *shermanii* strain P and *L. acidophilus* showed considerable inhibition against *B. cereus* but not against any other pathogenic bacteria.

Lactic acid and acetic acid present in the dips control the proliferation of pathogenic and spoilage organisms to a certain extent. It is suggested that the probiotic bacteria also produce these acids as a metabolic by-product which play a complementary role in inhibiting pathogenic and spoilage bacteria.

There were differences in the degree of the anti-fungal effects between the filter-sterilized bacterial metabolites (well diffusion assay) and live bacteria (spot-on-streak assay). When co-cultured together in broth media with probiotic bacteria, *L. paracasei* subsp. *paracasei* LCS1, *L. rhamnosus* strains GG and LR 1524 inhibited *S. cerevisiae. C. albicans* was controlled by *B. animalis* BB12, *L. paracasei* subsp. *paracasei* strains LCO1, LCS1 and all strains of *L. rhamnosus* (LC705, LBA, LGG and LR 1524) while *L. acidophilus* showed only a limited control on *C. albicans. P. freudenreichii* subsp. *shermanii* did not control *S. cerevisiae* but controlled *C. albicans* slightly. *S. cerevisiae* exhibited maximum level of resistance against the antagonistic effects of probiotic bacteria.

The inhibition of probiotic bacteria on moulds was the strongest against *Fusarium* spp., moderate against *P. roqueforti* and minimal against *A. niger.* However, pre-grown cultures of *P. freudenreichii* subsp. *shermanii* and *L. rhamnosus* strains GG and LR1524 showed moderate inhibition against *A. niger.* Out of all the bacteria tested, *L. rhamnosus* strain LR1524 produced larger quantities of acetic, lactic, butyric, benzoic and phenyl lactic acids and controlled spoilage and pathogenic bacteria yeast and moulds.

Pre-grown bacterial culture and/or their metabolites controlled yeast and mould more effectively than inoculating the bacteria cultures in commercially available forms (freeze-dried of frozen forms). The H_2O_2 produced by *L. acidophilus* created inhibitory zone against moulds. *L. acidophilus* also produced relatively larger quantities of benzoic and phenyl lactic acids compared with *L. casei* group bacteria. Large amount of lactic acid and moderate quantities of acetic, benzoic and phenyl lactic acids produced by *L. acidophilus* strain LAC1 are suggested to be involved in the control of moulds.

It is concluded that *L. acidophilus* strain LAC1, *P. fredenreichii* subsp. *shermanii* strain P and *L. rhamnosus* added in the form of late log phase or early stationary phase cultures are suitable bio-preservatives for acidic food like French onion dip in providing good protection against spoilage and pathogenic organism while adding the benefits of probiotics to consumers.

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CHAPTER 1
INTRODUCTION

1.1. Introduction

With the emergence of antibiotic resistant bacteria and natural ways of suppressing pathogens, the concept of 'probiotics' has attracted much attention. Probiotics are mono- or mixed- cultures of live microorganisms, which when introduced to human or animal, affect the host beneficially by improving the balance of the endogenous microflora of the gut (Fuller, 1993). Schaafsma (1996) re-defined 'probiotics', with emphasis to the microbial load of probiotics, as living organisms that upon ingestion in certain numbers exert health benefit beyond inherent basic nutrition. The Fermented Milks and Lactic Acid Beverages Associations of Japan has developed a standard which requires a minimum of 10^7 viable bifidobacteria cells ml^{-1} to be present in fresh dairy products (Ishibashi and Shimamura, 1993). Stanton *et al.* (2001) recommended that higher level (at least 10^6-10^7 g^{-1}) of viable probiotic bacterial cell is needed to provide health benefits.

A number of therapeutic benefits have been attributed to these organisms. Oliver *et al.* (1999) reported that a product was developed and marketed in Argentina with a selected culture of lactobacilli to control intestinal infections. The severity of diarrhea in children in day care centers in France was found to be controlled significantly by the consumption of milk fermented with *L. casei* (Pedone *et al.*, 1999). Shedding of rotavirus was reported to be reduced by the consumption of *B. bifidum* cells (Duffy *et al.*, 1994), *Lactobacillus* GG (Raza *et al.*, 1995), *L. casei* shirota strain (Saavedra *et al.*, 1995).

Many antimicrobial properties of the probiotic bacteria have been identified. Coconnier *et al.* (1998) reported an anti-microbial substance produced by a selected strain of *L. acidophilus* that was active against *Helicobacter pylori* both *in vivo* and *in vitro*. Recent literatures suggest that the production of large quantities of organic acids, inhibitory substances such as hydrogen peroxide and reuterin, bacteriocins and competitive exclusion of pathogens by occupying binding sites are some of the mechanisms by which probiotics control the intestinal niche. *L. casei, B. longum* and *Lactobacillus GG* are found to increase the body's immune response (Perdigon *et*

al., 1990b; Sutas 1996; Romond *et al.*, 1997). Many researchers have observed improved lactose utilization by lactose mal-absorbers. The characteristics such as microbial competition for ecological niches, and production of anti-microbial compounds such as bacteriocins, hydrogen peroxide, are also reported to be used in the preservation of food (Stiles, 1996).

The recent trend in food manufacture is to combine probiotics with prebiotics, which act as fermentable substrates for probiotics. Prebiotics are not digested by human enzymes but stimulate the growth and/or activity of one or a limited number of bacteria in the colon, thereby improving the host health (Conway and Wang, 1997). Prebiotics include oligosaccharides that are found in cholesterol lowering soy-based ingredients, therapeutic herbs, inulin and in vegetables such as onion, garlic and tomatoes (Heasman and Melbintin, 1999).

A number of food products including probiotic yoghurt, ice cream (Haynes and Playne, 2002), frozen fermented dairy deserts (Shah and Ravula, 2000), freeze-dried yoghurt (Rybka and Kailasapathy, 1995) and coleslaw (Rodgers and Odongo, 2002) have been employed as delivery vehicles for probiotics. The success in using a product as a delivery vehicle for probiotics depends on its ability to maintain required level of viable cells of the probiotics. Many factors, including the presence of hydrogen peroxide, high acid levels, inhibitory substances produced by yoghurt bacteria (Shah and Lankaputhra, 1997; Dave and Shah, 1997a), cell damage due to freezing (Lankaputhra and Shah, 1996a) and freeze drying (Rybka and Kailasapathy, 1995) or the presence of high content of oxygen (Shah and Lankaputhra, 1997) affect the level of viable cells of probiotics. Therefore, consumption of probiotics through a variety of products may improve the chances of ingesting and maintaining required level of viable probiotic cells, rather than relying on a single product. Dips could be a successful supplementary delivery vehicle for probiotic bacteria owing to its stable pH, buffering capacity of ingredients and the presence of prebiotics. However, there is no information available on the suitability of dips as a carrier of probiotic bacteria. The overall aim of the study was to establish the suitability of dips as a delivery vehicle for probiotics.

1.2. General aims and objectives

The general aim of this study was to establish the suitability of dips as a delivery vehicle for probiotic bacteria such as *Lactobacillus acidophilus, Lactobacillus casei, Lactobacillus rhamnosus, Bifidobacterium animalis* and *Propionibacterium freudenrenchii* subsp. *shermanii*. As a standard practice in the preparation of dips, a mixture of acetic acid, lactic acid and citric acid are used to bring the pH of the dips to appropriate levels. Oil and gums are also important ingredients to improve the texture of dips. These practices have potential implication to the survival of probiotics in dips. Shah (2000) suggested that simple and reliable methods for routine enumeration of probiotic organisms should be devised to ascertain the viability of probiotics during refrigerated storage and in the distribution chain.

Therefore, the initial experiment was aimed at selectively enumerating each of the commonly used probiotic bacteria (*Lactobacillus acidophilus, Lactobacillus casei, Lactobacillus rhamnosus* and *Bifidobacterium animalis*) the yoghurt bacteria (*Lactobacillus dlbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*) and *Propionibacterium freudenrenchii* subsp. *shermanii*. The second experiment evaluated the effects of dip conditions on the survival of the selected probiotic bacteria. The antimicrobial properties of the probiotic bacteria and the antimicrobial substances produced by them against pathogenic and spoilage bacteria and yeast and mould were evaluated in the third experiment.

1.3. Thesis outline

Chapter 2 contains the literature review related to the status of probiotics in food industry and the antagonistic properties and mechanisms of probiotics. Chapter 3 describes the development of a method and procedures adopted in the selective enumeration of yoghurt bacteria (*L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus*), probiotic cultures (*L. acidophilus*, *B. animalis*, *L. paracasei* subsp. *paracasei* and *L. rhamnosus*) and *Propionibacterium freudenrenchii* subsp. *shermanii*. This chapter has been published in full in the Journal of Dairy Science (Tharmaraj and Shah, 2003, J. Dairy Science 86: 2288-2296). The microenvironment of dip affecting the survival of probiotic bacteria is discussed in Chapter 4. This chapter has been accepted for publication by International Dairy Journal. The antimicrobial substances produced by probiotic bacteria against

pathogenic and spoilage yeast and mould and bacteria in dips are discussed in Chapter 5. Chapter 6 provides an overall conclusion on the prospects of using products like dips as a delivery vehicle for probiotic bacteria. Tables and figures are provided at the end of each chapter.

CHAPTER 2
LITERATURE REVIEW

2.1. Background

Dips are processed cheese and yoghurt-based supplementary foods available in ready-to-eat form. In theory, the potential of cheese and yoghurt-based dips as a carrier medium to deliver probiotics to their users in effective numbers is at least comparable to that of yoghurt. The ingredients used in dips include fresh vegetables and other material that could provide the indigestible fibers (prebiotics) that are used by probiotic bacteria for their growth and metabolism. As in many other foods, gums (xantham and CMC) are added to improve the texture of dips. The storage pH of dips is maintained at a relatively stable level at 4.0 to 4.4 throughout the shelf life. Therefore, dips can provide a stable, moderately acidic and suitable media containing prebiotics for delivery of probiotic microorganisms (e.g. *L. acidophilus, L. casei, B. animalis, L. rhamnosus* and *P. freudenrenchii* subsp. *shermanii*) in levels that are sufficient to provide therapeutic benefits for the users.

2.2. Probiotics and their significance in food

The concept of probotics evolved at the turn of the 20th century from a hypothesis first proposed by Noble prize winning Russian scientist Elie Metchnikoff (Bibel, 1988), who suggested that the long, healthy life of Bulgarian peasants resulted from their consumption of fermented milk products. Probiotics are originally defined by Fuller (1993) as mono- or mixed-cultures of live microorganisms, which when introduced to man or animal, affect the host beneficially by improving the properties of the endogenous micro-flora of the gut. Schaafsma (1996) re-defined probiotics as 'living organisms which, upon ingestion in certain numbers, exert health benefits beyond inherent basic nutrition'. Salminen *et al.* (1998) called it as live microbial food ingredients that have a beneficial effect on human health.

Mechanisms by which probiotics act in the prevention and control of diseases include stimulation of the immune system, improvement in food degradation and uptake within the bowel, microbial competition for ecological

niches, and production of compounds such as bacteriocins, bacterial metabolites, hydrogen peroxide and nitric oxide (Cleveland *et al.*, 2001). The function and metabolism of probiotics are discussed later in this section. The characteristics such as microbial competition for ecological niches, and production of anti-microbial compounds such as bacteriocins, hydrogen peroxide, are also reported to be used in the preservation of food (Stiles, 1996). The common probiotics used in foods are strains of *Lactobacillus* and *Bifidobacterium*. Although the classical strains of *Propionibacterium* have been in use in the dairy industry for centuries, the probiotic potentials of these bacteria has been exploited only recently (Korneyeva, 1981; Lehto and Salminen, 1997).

Many strains of *Lactobacillus* and *Propionibacterium* are found to inhibit pathogens and spoilage organisms in foods including milk, sauerkraut and vacuum packaged meats and fish (Al-Zoreky *et al.*, 1993; Leroi *et al.* 1996). Fiorentini *et al.* (2001) found that neutralized supernatants of *Lactobacillus plantarum* strain BN extended shelf life of raw bovine meat by inhibiting psychrotrophic and mesophilic aerobic microorganisms. Vascovo *et al.* (1995) found that inoculation of salads with *L. casei* or *Pediococcus pentosaceus* resulted in domination of the vegetables with these bacteria and a dramatic decrease in entero-bacteria compared to un-inoculated control samples.

L. acidophilus, L. casei, L. paracasei, L. rhamnosus, L. plantarum, L. reuteri and *L. salivarius* are some of the lactobacilli recognized as probiotic bacteria and *Bifidobacterium lactis, B. longum* and *B. breve* are some of the bifidobacteria strains used as probiotics (Yeung *et al.*, 1999). *L. acidophilus, L. casei, L. rhamnosus* and *Bifidobacterium* are normal inhabitants of the intestine of humans and animals. They belong to the group of bacteria called lactic acid bacteria (LAB) which are Gram negative, non spore forming and non respiring organisms with rod, cocci, branched or amorphic morphology.

2.3. Carbohydrate metabolism of probiotic bacteria

In most LAB, lactic acid is the major metabolic by-product of carbohydrate metabolism. The LAB can be grouped as homo-fermentative or hetero-fermentative, based on their pattern of carbohydrate fermentation. The LAB which produce lactic acid as their major by-product are referred to as homo-fermentative and those which

produce CO_2, ethnol and acetic acid as their major by-products in addition to lactic acid are referred to as hetero-fermentative.

Homo-fermentative LAB follows Embden-Myerhof –Parnas (EMP) pathway for glycolysis to produce D-, L- or a racemic mixture of DL- lactic acid. There are four pathways associated with the hexose metabolism in LAB (Ray and Daeschel, 2000). Pathways associated with hexose metabolism in LAB include Embden-Meyerhof-Pranas pathway, hexose mono-phosphate shunt, mixed acid fermentation and pentose- phosphate pathway. Bifidus pathway is associated with bifidobacteria and succinate- propionate pathway with propionibacteria. In homo-fermentative fermentation, glucose and other usable hexoses are converted mainly to lactic acid through the EMP pathway, when the carbohydrate supply is unlimited. Each molecule of glucose is potentially converted to 2 molecules of lactic acid (Figure 2.1).

Species from lactococcus, streptococcus (*S. thermophilus*), pediococcus and groups I and II *Lactobacillus* metabolize hexose through the EMP pathway. However, in limiting concentrations of hexose, some strains of *L. lactis* spp. *cremoris, S. thermophilus* and some group II lactobacilli (*L. casei* group) can produce acetate and CO_2 from pyruvate. *Leuconostocs* and group III lactobacilli, however, metabolize hexose through hexose mono phosphate shunt (HMS) with production of equimolecular amounts of CO_2, acetate and lactate (Figure 2.2).

However, the production of acetate is reduced under a limited oxygen environment, since under these conditions acetyl-phosphate will be diverted to ethnol (Figure 2.2). In limiting hexose concentration, leuconostos use a different pathway (mixed acid pathway) to generate formate along with acetate and lactate (Figure 2.3).

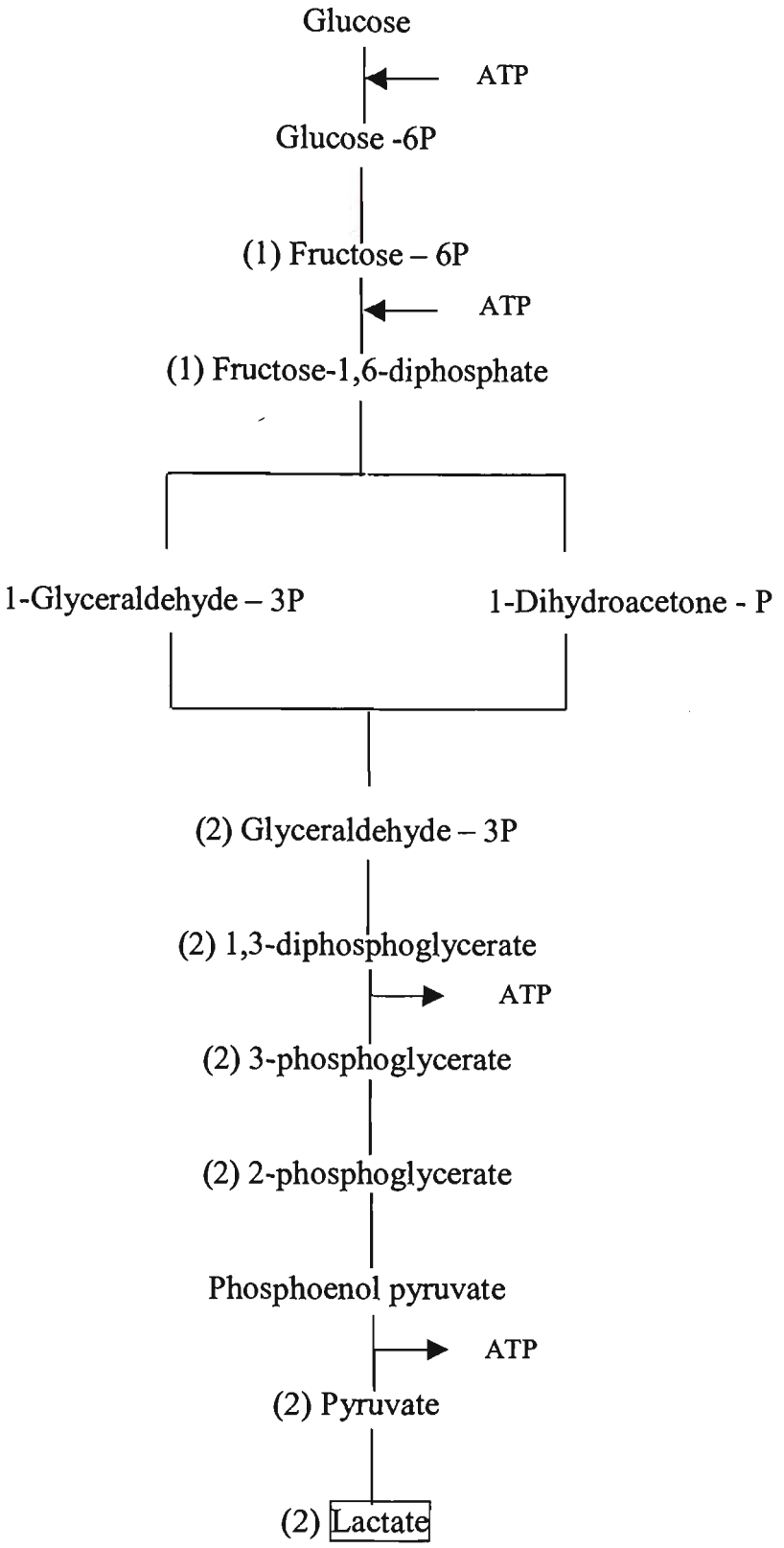

Figure 2.1 Embden-Myerhof-Parnas pathway for glycolysis of homo-fermentative LAB (Source: Ray and Daeschel, 2000)

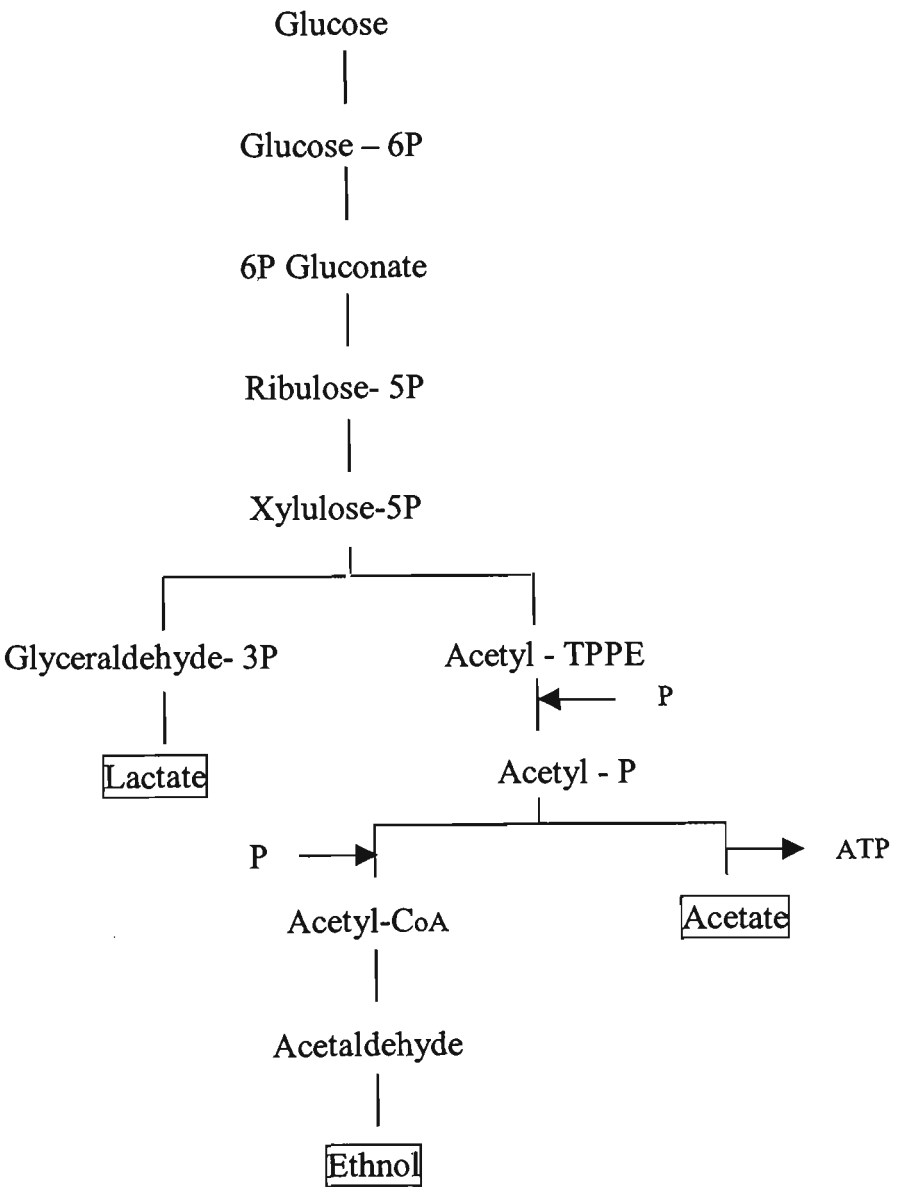

Figure 2.2 Hetero-lactic fermentation of hexose by hexose monophosphate shunt (HMS) (Source: Ray and Daeschel, 2000)

Those species capable of fermenting pentose sugars do so through the pentose-phosphate pathway (Figure 2.4), with the production of equimolecular amounts of acetate and lactate. Again the conversion of acetyl-P to either acetate or ethnol is regulated by the availability of oxygen and the availability of necessary enzymes. In all these pathways in general more lactate than acetate (on a molecular basis) is produced. Pyruate, intermediately formed in the above mentioned pathways may partially undergo several alternative conversions, yielding either the well-known aroma compound diacetyl and its derivatives, or acetic acid (ethnol). Even

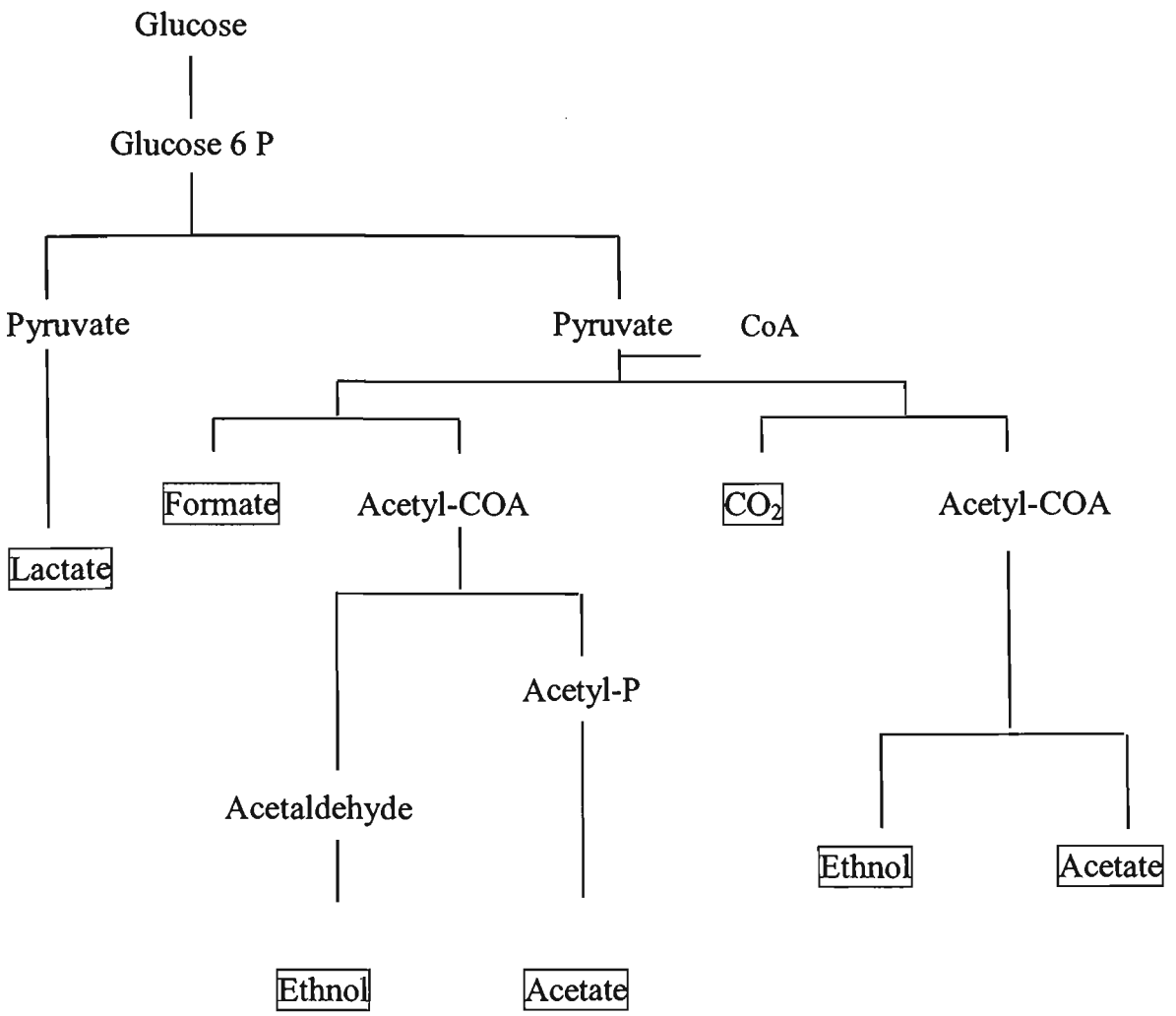

Figure 2.3 Mixed acid fermentation in limiting hexose concentration by some LAB (Source: Ray and Daeschel, 2000)

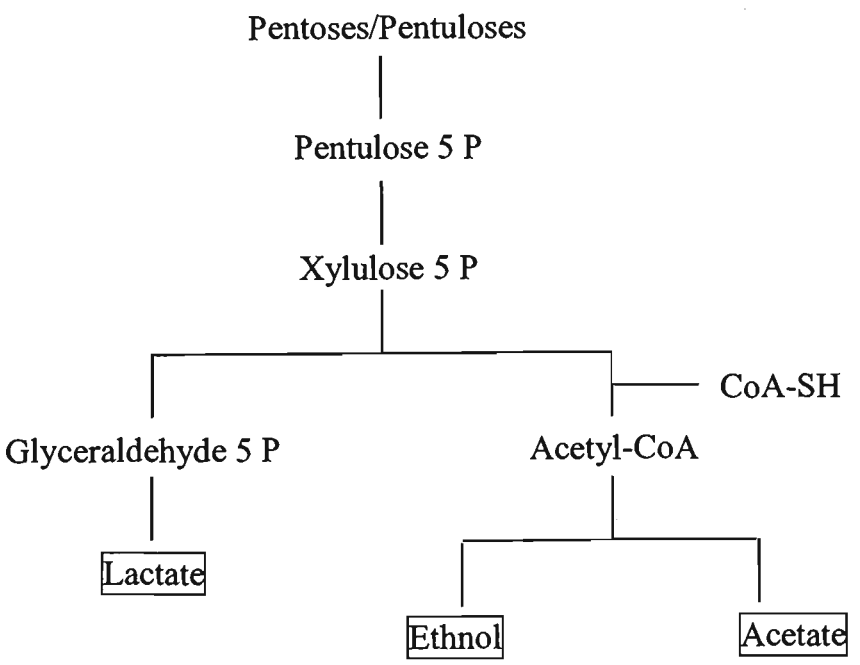

Figure 2.4 Pentose- phosphate pathway (Source: Ray and Daeschel, 2000)

lactate may partially be oxidized and broken down to acetic acid and formate or CO_2 (Kandler and Weiss, 1986).

In contrast *Bifidobacterium* species produce more acetate than lactate (3:2) from hexose by bifidus pathway (Figure 2.5).

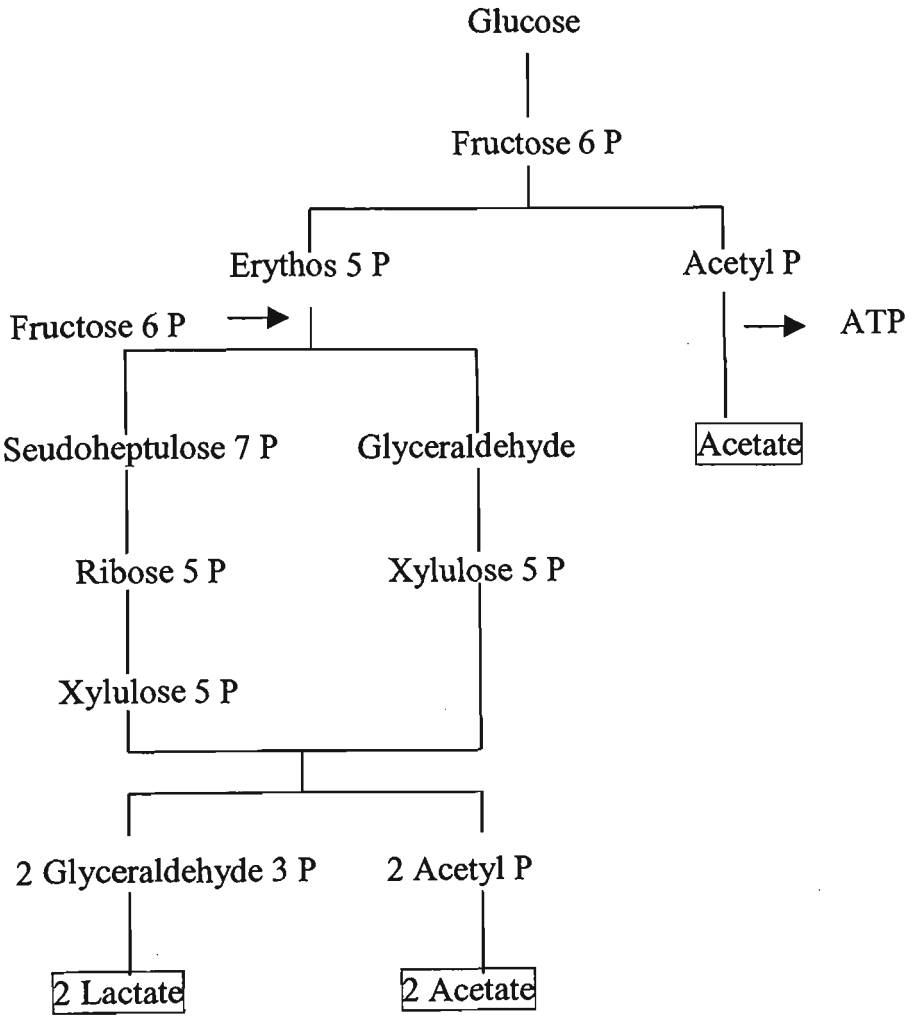

Figure 2.5 Bifidus pathway (Source: Ray and Daeschel, 2000)

Propionibacterium are capable of converting pyruvate to produce considerable amounts of propionic acid along with some acetic acid and CO_2 through succinate-propionate pathway (Figure 2.6).

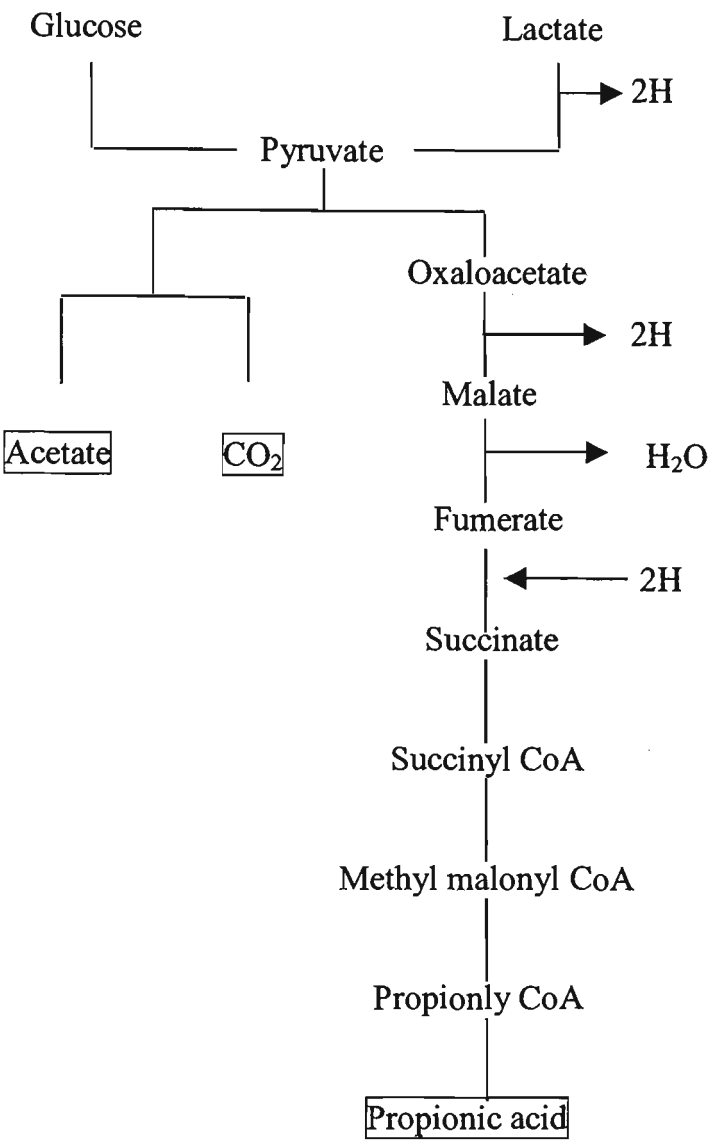

Figure 2.6. Succinate-propionate pathway (Source: Ray and Daeschel, 2000)

2.4 Taxonomic diversity of probiotic bacteria

2.4.1 Genus Lactobacillus

Lactobacilli are found in association with substrate, rich in various carbohydrates and thus in a variety of habitats such as mucosal membrane of humans and animals, mainly oral cavity, intestine or vagina or on plant material and fermenting food (Pot *et al.,* 1994). Lactobacilli are strictly fermentative, aero-tolerant or anaerobic, aciduric or acidophilic and have complex nutritional requirement. With glucose as a carbon source lactobacilli could be either homo-fermentative or hetero-fermentative.

When homo-fermentative, they produce more than 85% of lactic acid and the hetero-fermentative strains produce lactic acid, CO_2, ethnol or acetic acid in equimolecular quantities. In the presence of oxygen or other oxidants, increased amounts of acetate may be produced at the expense of lactate or ethnol. Lactobacilli contain no isoprenoid quinons except *L. yamanashiensis* and *L. casei* subsp. *rhamnosus* and no cytochrome systems to perform oxidative phosphorylation (Kandler and Weiss, 1986). However, they possess flavin containing oxydases and peroxidases to carry out the oxidation of $NADH_2$ and O_2 as the final electron acceptor. They are also able to perform a manganese catalyzed scavenging of super oxide, although they do not possess super oxide dismutase or catalase.

L. salivarius may be the most typical species of the mouth flora, although it is also found in the intestinal tract (Kandler and Weiss, 1986). The most prominent species, probably indigenous to the intestine is *L. acidophilus*, which is believed to exert a beneficial effect on human and animal health. It is used in industrial scale in preparing acidophilus sour milk and producing pharmaceutical preparations to restoring the normal intestinal flora after disturbance caused by disease or treatment with antibiotics (Kandler and Weiss, 1986).

2.4.2. L. acidophilus

These are rods with rounded ends, generally 0.6-0.9 x 1.5-6.0 μm, occurring singly, in pairs and in short chains. These bacteria ferment carbohydrates such as cellobiose, esculin, fructose, galactose, glucose, lactose, maltose, mannose, salicin, sorbitol and sucrose. Starch is fermented by most strains. With rare exceptions, they show good growth at 45° C (Kandler and Weiss, 1986).

2.4.3. L. casei

L. casei are Gram positive, facultively anaerobic, non-motile and non-spore forming rod shaped (cell size range, 0.7-1.1 x 2.0- 4.0 μm) members of the industrially important lactic acid bacteria. They are acid tolerant, cannot synthesize porphyrins and posses a strictly fermentative metabolism with lactic acid as the major metabolic end product (Axelson, 1998; Kandler and Weiss, 1986). Within the genus *Lactobacillus*, *L. casei* form part of the facultatively heterofermentative (group ll) species cluster, which produce lactic acid from hexose sugars via the Emden-

Myerhof pathway and from pentoses by the 6-phosphogluconate/ phosphoketolase pathway/ pentose- phosphate pathway (Axelson, 1998). Growth of these bacteria occurs at 15°C but not at 45°C, and requires riboflavin, folic acid, calcium pantothenate and niacin growth factors (Kandler and Weiss, 1986). They are found in raw and fermented dairy products, fresh and fermented plant products and the reproductive and intestinal tracts of humans and other animals (Kandler and Weiss, 1986). The 9th edition of Bergey's Manual of Systemic Microbiology recognized 4 species of *L. casei*: *L. casei* subsp. *casei, L. casei* subsp. *pseudoplantarum, L. casei* subsp. *rhamnosus,* and *L. casei* subsp. *tolerans* (Kandler and Weiss, 1986). However, more detailed phylogenetic studies have led to proposals that members of the *L. casei* group be divided into three species: *L. rhamnosus, L. zeae* and *L. casei* (Chen *et al.*, 2000; Dellaglio *et al.*, 1919; Dicks *et al.*, 1996)

2.4.4. L. rhamnosus

These organisms are the only homo-fermentative lactobacilli that grow well at both 15° C and 45° C (Kandler and Weiss, 1986). Nutritional requirements are similar to that of *L. casei.* But this species ferment rhamnosus that is not fermented by *L. casei* (Kandler and Weiss, 1986). Strains of this bacterium are popular as bio-preservative (*L. rhamnosus* LC705) and as probiotics (*L. rhamnosus* GG or LGG). LGG was the first probiotics proven to colonize the GI tract (Goldin *et al.,* 1992)

2.4.5. Bifidobacteria

Bifidobacteria are nonmotile, nonsporing, Gram positive rods of varied shapes that are slightly curved and clubbed and often branched. The rods can be single or in clusters and V shaped pairs. *Bifidobacterium* spp. is strictly anaerobic microorganisms. However, the degree of tolerance of oxygen depends on the species and culture medium (Ballongue, 1989). Three types of responses are observed during the switch from anaerobiosis to aerobic conditions:

1. Aerobic growth without the accumulation of H_2O_2
2. Limited growth with the accumulation of H_2O_2 that is considered to be toxic for the key enzyme in the sugar metabolism of bifidobacteria: fructose-6-phosphate phosphoketolase

3. No growth without the accumulation of H_2O_2. These strains require low redox potential for growth and fermentation.

The presence of NADH oxidase helps these bacteria to convert the oxygen to hydrogen peroxide, which is subsequently reduced by NADH peroxidase to non-toxic forms. The strains most sensitive to oxygen had low NADH peroxidase activity, resulting in an accumulation of toxic hydrogen peroxide (Ballongue,1989).

The optimum temperature for the development of the human species is between 36 and 38°C. In contrast, that for the animal species is slightly higher, about 41-43°C and may even reach 46.5°C. There is no growth below 20°C and these bacteria have no thermo resistance above 46°C (Scardovi, 1986; Ballongue, 1989). Optimum growth pH is between 6.5 and 7.0. No growth can occur below 5.0 or above 8.0.

Bifidobacteria actively ferment carbohydrates to produce acetic and lactic acid, but no CO_2 through bifidus pathway. Smaller quantities of formic acid and ethnol are formed by the splitting of pyruvate. It is found in the mouth and intestinal tract of warm-blooded vertebrates, sewage, and insects. *B. bifidum* is a pioneer colonizer of the human intestinal tract, particularly when babies are breast fed. A few *Bifidobacterium* infections have been reported in humans (Prescott *et al.*, 1999). Species of bifidobacteria produce considerable quantities of vitamins such as thiamin (B1), riboflavin (B2), Pyridoxine (B6), folic acid (B9), cyanocobalamine (B12) and nicotinic acid (pp) (Balongue, 1989). Most bifidobacteria are resistant to numerous antibiotics and notably to nalidixic acid, gentamycin, kanamycin, metronidazole, neomycin, polymyxin B and streptomycin but the sensitivity of the species varies from 10-500 $\mu g.mL^{-1}$ or more. In contrast, ampicillin, bacitracin, chloramphenicol, clindamycin, erythromycin, lincomycin, nitrofurantoin, oleandomycin, penicillin G, and vancomycin strongly inhibit most species (Scardovi, 1986; Ballongue,1989).

2.4.6. Propionibacteria

They are pleomorphic rods, 0.5-0.8 x 1-5 μm often diptheroid or club shaped with one end rounded and the other end tapered or pointed. However, cells may be coccoid, bifid or even branched. Cells may occur singly, in pairs or short chains, in V or Y configuration, or in clumps with Chinese character arrangement. Gram

positive, non-motile, non-sporing chemoorganotrophs fermentation products include large amounts of propionic acid and acetic acid, and generally lesser amounts of iso-valeric, formic, succinic or lactic acids and carbon dioxide. Anaerobic to aero-tolerant, generally catalase-positive. Growth is most rapid at 30- 37°C and the colony may be white, gray, pink, red, yellow or orange in colour (Cummins and Jonson, 1986). The genus *Propionibacterium* is split into 'cutaneous' and 'dairy' (or "classical). The 'dairy' propionibacteria play an important role as a biopreservative and probiotic in food and therefore is discussed here.

The 'dairy' propionibacteria can be isolated from dairy foods and silage. These organisms are used commercially in the production of propionic acid (Grant and Salminen, 1998) and in the production of vitamin B12 (Medigan *et al.*, 2000). Medigan *et al.* (2000) further stated that *Propionibacterium* give yields of this vitamin ranging from 19-23 mg/L in a two-stage process. This bacterium also takes part in the production of flavour compounds in cheese by proteolysis and propionic acid (Dupis *et al.,* 1995; Østile *et al.,* 1995). Dairy propionibacteria are autolytic under the environmental conditions found in cheese and degrade peptides and aminoacids that are present in the cheese. This activity increases the amount of free prolin in the cheese, aiding flavor development (Dupis *et al.,* 1995; Østile *et al.,* 1995).

The classical propionibacteria were divided into variety of species using phenotypic properties but these have been grouped into 4 as the result of DNA/DNA homology studies. The species are *Propionibacterium jensenii, P. theonii, P. acidipropionici* and *P. freudenreichii*. Propionibacteria are Gram positive, anaerobes that ferment lactic acid, carbohydrates, and polyhydroxy alcohols producing primarily propionic acid, acetic acid and CO_2. Their nutritional requirements are complex and they usually grow rather slowly. When metabolizing glucose, the initial catabolism of glucose to pyruvate follows the EMP pathway as in LAB then follows the succinate- propionate pathway. Propionibacteria ferment lactate with the production of propionate acetate and CO_2. This metabolic strategy is called the secondary fermentation and this reaction is important in the production of Swiss cheese.

2.5. Activities of probiotic bacteria

Consumption of *B. longum* strain BB 536 has effected an alleviation of constipation, prevention of diarrhoea, protection from infection, prevention of cancer and enhancement of calcium absorption measured in terms of an increase in bone density (Balongue *et al.*, 1993; Ogata *et al.*, 1999). Ogata *et al.* (1999) reported that the administration of yoghurt containing *B. longum* BB 536 has improved the intestinal environment while reducing putrefactive substances and urease activity. The high numbers of *E. coli*, bacteriods, and *Candida* found in the feces of patients affected by leukemia were reduced by administration of the cultured milk drink, Morinaga Bifidus containing BB536 (Kageyama *et al.*, 1987; Tomoda *et al.*, 1988) without any noticeable harmful effects.

When consumed in yoghurt, bifidobacteria (Colombel *et al.*, 1987) reduced the course of erythromycin induced diarrhoea and *Lactobacillus* GG (Siitonen *et al.*, 1990) reduced the diarrhoeal phase as well as the side effects (such as abdominal distress, stomach cramps, and flatulence) and colonized the bowel. Other proven beneficial effects of *Lactobacillus rhamnosus* GG include promoting systemic and local immune response to rotavirus (Kaila *et al.*, 1992), reducing hepatic-encephalopathy, a condition caused by the increase of blood ammonia in patients with liver disorder (Lehto and Salminen, 1996), reducing constipation (Lehto and Salminen, 1996) and suppressing the bacterial enzyme activity that increase the risk of colon cancer. Oral introduction of *Lactobacillus rhamnosus* GG has been associated with alleviation of intestinal inflammation and normalisation of increased intestinal permeability (Grant and Salminen, 1998). Grant and Salminen (1998) further suggested that it may be of benefit to have propionibacteria present in the gut because propionibacteria can utilise lactate produced by bifidobacteria, lactobacilli and other bacteria, since propionibacteria benefit the host by producing propionic acid, a short chain fatty acid.

Salminen *et al.* (1998) suggested that *P. freudenreichii* could be used as a probiotic because it is resistant to gastric digestion *in vitro*. Salminen *et al.* (1998) further showed that *P. freudenreichii* spp. *shermanii* JS bound to cultured Caco-2 intestinal epithelial cells in similar levels to *L.* GG, an isolate known to bind well to Caco-2 intestinal epithelial cells. But when treated with *L. rhamnosus* LC-705

(DSM 7061) only smaller number of *P. freudenreichii* spp. found to be adhered indicating competition.

The criteria used in the selection of probiotic organisms include safety, functionality (e.g. survival, adherence, colonization, anti-microbial production, immune stimulation, antigenotoxic activity and prevention of pathogens) and technological aspects (e.g. growth in milk or in other food bases, sensory properties, stability, phage resistance and viability) (Salminen *et al.*, 1998).

2.5.1. Safety concerns in the use of probiotics in food

Safety of a food product when a microorganism is included becomes the main concern of producers as well as for the consumers since the popular picture the microorganisms bring to memory are their pathogenic effects. Food grade lactic acid bacteria have gained an important ecological role in food preservation and have a good record of safety (Adams and Marteau, 1995). Adams and Marteau (1995) further stated that the overall risk of LAB infection is very low, particularly in view of their ubiquity in the environment. Acute toxicity studies conducted by Donohue *et al.* (1993) using several LAB and *B. longum* for reference have shown no acute toxicity (Table 2.1).

Table 2.1. Acute toxicity of probiotic bacteria (adapted from Donohue *et al.*, 1993)

Probiotic strain	LD_{50} (g/kg body weight)
Streptococcus faecum AD 1050 [a]	>6.6
Streptococcus equinus [a]	>6.39
Lactobacillus fermentum AD002 [a]	>6.62
Lactobacillus salivaricus AD0001 [a]	>6.47
Lactobacillus GG (ATCC53103)	>6.00
Lactobacillus helveticus	>6.00
Lactobacillus bulgaricus	>6.00
Bifidobacterium longum	25.00

[a] heat treated nonviable preparations.

The safe-to-use probiotics as suggested by Donohue and Salminen (1996a) are listed in Table 2.2. Twenty-four species of *Bifidobacterium* have been recognised

Table 2.2 Safety studies and reported effects of current successful probiotic and yoghurt strains

Probiotic strain	Reported effects	Method of safety study		
		in-vitro	*in-vivo*	in human
L. *acidophilus* NFCO1748	Treatment of constipation, alleviation of radiotherapy-related diarrhoea, lowering of faecal enzymes	+	+	+
L. casei spp. *Shirota*	Balancing intestinal microflora, prevention of intestinal disturbances, treatment of superficial bladder cancer	+	+	+
Lactobacillus GG (ACTT 53103)	Treatment of acute viral and bacterial diarrhoea in infants, prevention of antibiotic-associated diarrhoea, immune enhancing, and stabilisation of intestinal permeability	+	+	+
L. acidophilus (johnsonii) LC1	Immune enhancing, vaccine adjuvant, balancing intestinal microflora	+	+	+
Bifidobacterium bifidum	Prevention of rotavirus diarrhoea	+	+	+

Source: Donohue and Salminen (1996a)

so far, out of which nine species (*B. bifidum, B. longum, B. infantis, B. breve, B. adolesentis, B. angulatum, B. catenulatum, B. psudocatenulatum* and *B. dentium)* were human-derived. Ballonge (1998) reported that *B. bifidum, B. breve, B. infantis,* and *B. longum* are safe to be used in foods and do not constitute any danger. However, Ballonge (1998) cautioned that *B. dentium* can be recognised as being pathogenic, and that the other four strains of bacteria mentioned above could be confused with *B. dentium,* if the identification of the strain used is not done using genetic methods.

Propionibacterium is classified into two groups, the classical or dairy strains and the cutaneous or human strains. The classical strains of *Propionibacterium* have been safely used in cheese making for centuries. The human strains are usually associated with pathogenesis (Swidsinski *et al.,* 1995; Brook, 1994; Debelain *et al.,* 1995). Funke *et al.* (1997) suggested that it is likely that the presence of these organisms in the disease is opportunistic. The dairy (or classical) strains of

Propionibacterium (*P. acidipropionici, P. freudenreichii, P. jensenii* and *P. theoni*) are widely recognised as food grade organisms, and are widely used as starter culture in the production of cheese. Status as a " safe food grade organism" should be a primary prerequisite in using a given genus as a probiotic for humans.

2.5.2 Functional aspects of probiotics

Functional aspects of probiotic bacteria such as survival, adherence, colonization, immune stimulation, prevention of pathogen and anti-microbial activity are important characteristics to be considered when selecting probiotics for use (Salminen *et al.*, 1998). Table 2.3 provides some mechanisms of probiotic functionality and its beneficial effects.

Table 2.3 Mechanism of probiotic functionality and its beneficial effects (adapted from Salminen *et al.*, 1998)

Mechanism of functionality	**Beneficial effects**
Anti-microbial activity	Control of rotavirus and *Clostridium difficile* Control of ulcers related to *Helicobacter pylori* Antibiotic therapy Treatment of diarrhea associated with travel
Colonization resistance	Balancing of colonic micro-biota
Immune effects Adjuvant effect	Vaccine adjuvant effect
Cytokinine expression	Enhanced immune response
Stimulation of phagocytosis by peripheral blood leucocytes Secretory IgA	Enhanced immune response
Influence on enzyme activity	Reduction of fecal enzymes implicated in cancer initiation Reduction in serum cholesterol
Enzyme delivery	Amelioration of lactose malabsorption
Anti-mutagenic effects	
Anti-genotoxic effects	

A high intake of fermented milk products was associated with decreased risk of ulcer, whereas an increased risk of ulcer was noted with high intake of milk (Elmsthal *et al.,* 1998). Gismondo *et al.* (1990) demonstrated that *L. acidophilus* and *B. bifidum* in concentrations of 10^9 bacteria act as an "ecological" therapy for gastritis and duodenitis. A fermented product containing *L. acidophilus* has been shown to inhibit the growth of pathogenic organisms like *S. dysenteriae, S. typhosa* and *E. coli.* They further suggested that the beneficial effect of feeding in bacterial diarrhea might be due to the anti-microbial metabolites produced by *L. acidophilus*, which might have neutralized the entero-toxins of *E. coli.* Silva *et al.* (1999) also observed similar pattern with bifidobacteria when Bifidus milk was fed, the protection against *S. enteritidis* subsp. *typhimurium* was not due to the reduction of intestinal population of pathogenic bacteria.

In cases of *H. pylori* infection, Kabir *et al.* (1997) indicated that *L. salivarius,* but neither *L. casei* nor *L. acidophilus,* proved to be capable of producing high amounts of lactic acid and thus completely inhibiting the growth of *H. pylori* in a mixed culture. Further *L. salivarius* inhibited both attachment and IL-8 release in vitro and that *H. pylori* could not colonize the stomach of *L. salivarius* infected gnotobiotic BALB/c mice but colonized in large numbers and caused active gastritis in germ free mice (Bazhenov *et al.,* 1997). Kabir *et al.* (1997) found that *L. salivarius* given after *H. pylori* implantation could eliminate colonization by *H. pylori.* Bazhenov *et al.* (1997) found that the presence of high antagonistic activity in strains of *L. casei* 925, *L. plantarum* 8RA-3, *L. fermentum* BL-96 and L. 90265 against *H. pylori.* Midolo *et al.* (1995) found that a strain of *L. casei* subsp. *rhamnosus* inhibited the growth of *H. pylori.* Jiang *et al.* (1997) suggested that lactobacilli such as *L. acidophilus* strain LA1 improves lactose digestion by improving *in vitro* lactose fermentation.

Human-derived strains of LAB and *Bifidobacterium* spp. are preferred for their beneficial function (Salminen *et al.,* 1998; Ballongue, 1998). *L. acidophilus* NCDF 1748, *Lactobacillus* GG (ATCC 53103) *L. reuteri, L. rhamnosus* (LA705) and *L. acidophilus* BG2F04 have been shown to be adherent to Caco-2 cell or in other systems (Lehto and Salminen, 1996, 1997). *Lactobacillus* GG (ATCC 53103), *L. reuteri, L. gasseri ADH* and *L. acidophilus* LA1 are found to colonise the intestinal

tract (Saxelin *et al.,* 1995; Goldin *et al.*, 1992). Noricatsu *et al.* (1999) showed that *L. casei* strain Shirota survived transit through the gastrointestinal tract after ingestion of fermented milk with this organism. Dairy *Propionibacterium* strains were also found to colonise the digestive tract of humans (Korneyeva, 1981). Mitsuoka (1989) has shown that bifidobacteria can colonise the intestinal tract. The above literature suggests that the strains of LAB, *Bifidobacterium* spp. and *Propionibacterium* spp. can survive and colonise the intestinal tract successfully to exert probiotic effects.

The beneficial effects of *Lactobacillus* and other probiotics have also been attributed to their ability to suppress the growth of pathogen probably by secretion of anti-microbial substances such as lactic acid, hydrogen peroxide, and bacteriocins (Shah and Dave, 2002). The LGG strain was shown to produce an anti-microbial substance with a broad spectrum of activity against a range of bacteria (Biadaioli and Rubaltelli, 1998).

2.5.2.1. Probiotics as bio-preservatives

The anti-microbial substances produced by LAB and *Propionibacterium* are also used in the bio-preservation of food (Stiles, 1996). LAB is used in bio-preservation because they naturally dominate the micro-flora during the storage of many foods. They are used in the preservation of milk products, brined vegetables, cereal products and in vacuum packaged meat (Stiles, 1996). Propionibacteria have been used for flavour enhancement in cheese (Dupuis *et al.*, 1995) and in bio-preservation of food (Al-Zoreky *et al.*, 1993). Preservation of food by biological methods has originated with the use of fermentation to produce wine, vinegar, yoghurt, cheese, butter and bread long before biblical age when civilization entered into the metal age.

Bio-preservatives are anti-microbial compounds that are of plant-, animal- or microbial-origin that does not have any adverse effect on human health. Fermented foods are good example of bio-preserved foods in which the starter cultures are allowed to grow in order to produce anti-microbial metabolites. Nisen-Meyer and Nes (1997) suggested that, to maintain their existence or ecological niche, many bacterial species have developed an anti-microbial defense system against competitors or infections. Microorganisms of genera *Lactococcus*, *Lactobacillus*,

Leuconostoc, *Streptococcus*, *Pediococcus*, *Enterococcus* and *Carnobacterium*, probiotic bacteria that are of human origin such as *L. acidophilus*, *L. casei*, *L. rhamnosus*, *Bifidobacterium* spp. and dairy strains of *Propionibacterium* are reported to produce anti-microbial compounds (Conway, 1996; Dayl and Davis, 1998; Hugas, 1998).

Organic acids, short chain fatty acids, hydrogen peroxide, reuterin, diacetyl, bacteriocins and bacteriocin-like inhibitory substances are some of the metabolic products of these bacteria, suggested to have potential anti-microbial effects (Holzapfel *et al.*, 1995; Ouwehand, 1998; Ray and Daeschel, 1992; Cleveland *et al.*, 2001; Shah and Dave, 2002). Inhibition by the anti-microbial metabolites, competition for nutrition and niche and altered redox potential are some of the ways in which the pathogenic and spoilage organisms are inhibited. Many anti-microbial agents have been in use for a long time without any known adverse effects. For example, many of the organic compounds used in the food industry are also anti-microbial metabolites of bacteria associated with fermented food products. Lactic acid produced by the starter culture in yoghurt prevents the growth of undesirable microorganisms (Ray and Daeschel, 1992).

Organic acids such as lactic and acetic acids produced by lactic acid bacteria help to lower the pH and create unfavourable environment for other organisms, including pathogenic and spoilage organisms. Hydrogen ion was widely believed to be associated with the anti-microbial effect. Recently, the bacterio-static and bactericidal effects of weak acids are found to be caused by the un-dissociated molecules of these acids, rather than the hydrogen ion. The un-dissociated acid molecules damage the pathogens through acidification of cytoplasm, destruction of the trans-membrane proton motive force, loss of active transport of nutrient through the membrane and by causing sub-lethal injury (Booth and Kroll, 1989; Brown and Booth, 1990; Kabara and Eklund, 1990; Shah and Dave, 2002).

The concentration, pH, pKa, lipophilic property and solubility of the acids, the micro-environmental temperature and the microbial load of the media influence the anti-microbial effect of these acid (Brown and Booth, 1990; Kabara and Eklund, 1990). Shah and Dave (2002) indicated that some strains of LAB including Lactococci, Lactobacilli, Leuconostocs, and Pediococci have the ability to produce hydrogen peroxide but do not catalyze it, thereby acquiring a protection by the

accumulated hydrogen peroxide in the growth media. These authors further stated that hydrogen peroxide inhibits the growth of *S. aureus, E. coli, Salmonella typhimurium, Clostridium perfringens, Pseudomonas* spp. and other psychrotrophs. *L. reuteri* (previously classified as *L. fermentum*) produces reuterin during glycerol metabolism. Reuterin is active against a broad spectrum of Gram positive and Gram – negative bacteria (Talarico *et al.*, 1988; Axelson *et al.*, 1989; Chung *et al.*, 1989; Nakanishi, 2002) and fungi (Magnusson and Schnurer, 2001). Most lactic acid bacteria produce diacetyl (2,3-butanedione) during the stationary growth phase by metabolizing the pyruvate accumulated during the exponential growth phase.

Some citrate fermenting bacteria such as *Lactococcus lactis* subsp. *lactis* var. *diacetylactis* and *Leuconostocs* spp. produce diacetyl through the fermentation of citrates. Diacetyl shows a broad-spectrum anti-microbial activity against Gram negative and Gram positive bacteria and yeast and mould (Ray and Daeschel, 1992). Dieleveux *et al.* (1998) attributed phenyllactic acid to the inhibition of various pathogenic bacteria such as *L. monocytogenes, S. aureus, E. coli* and *Aeromonas hydrophila*. Above all phenyllactic acid has been reported to be one of the most abundant aromatic acids to which anti-microbial properties have been attributed and occurs in several honeys with different geographical origins (Steeg and Montag 1987; Weson *et al.*, 1999).

Propionic acid inhibits the growth of fungi and bacteria. Propionate is used in the manufacture of bread because it inhibits spoilage organisms, and suppresses "ropiness" which may be caused by organisms such as *Bacillus mesentericus* (Luke, 1980). For such uses propionic acid and propionates are accepted food additives in most countries of the world. Propionibacteria are found in sourdough starters aiding the development of flavour and are associated with the inhibition of spoilage organisms. The shelf life of bread can be extended by 2-4 days by the use of *Propionibacterium* cultures and lactic acid bacteria. Microgard, a preparation of metabolites from *Propionibacterium shermanii* is inhibitory against Gram negative organisms. Its activity has been attributed to a bacteriocin and to a lesser extent the presence of organic acids such as propionic acid and acetic acid (Al-Zoreky et al., 1993; Grant and Salminen (1998). Another commercial product, Bioprofit which contains *L. rhamnosus* LC-705 (DSM7061) and *P. freudenreichii* spp. *shermanii* JS strains. Also combinations of *L. rhamnosus* LC-705 (DSM7061) and *P.*

freudenreichii subsp. *shermanii* JS have been used as silage inoculants for preservation and inhibition of moulds and yeasts. Inoculation with the combination decreased the growth of enterobacteria, clostridia and fungi when compared with silage without additives (Grant and Salminen, 1998).

2.5.2.2. Inhibitory effects of probiotic and lactic acid bacteria on yeast and mould

Reports on food poisoning caused by fungus dates from the 10th century with Ergotism or St Anthony's fire or 'Holy fire caused by toxin produced by the fungi *Claviceps purpurea* on cereals (Pohland, 1993; Van Dongen 1995; Packers 1998). In the mid 20th century, another fungal toxin aflatoxin which has strong carcinogenic properties was reported to be produced by the fungus *Aspergillus flavus* (Filtenborg, *et al.*, 1996). Today, more than 400 myco-toxins are known from many different genera and the number is increasing rapidly (Filtenborg *et al.*, 1996).

In addition to health hazard from myco-toxins, yeast and mould cause considerable spoilage of food. It is estimated that between 5-10% of world's food production is lost due to fungal deterioration (Pitt and Hocking, 1999). *Aspergillus* and *Penicillium* species have been reported as spoilage organisms during storage of a wide range of food products. Species of fungi *Fusarium* are often found on cereal grains where they may produce a number of myco-toxins (Filtenborg *et al.*, 1996; Samson *et al.*, 2000). *P. roqueforti* commonly spoil hard cheese. Many strains of yeast are important spoilage organisms of yoghurt and other fermented food products (Pitt and Hocking, 1999).

Many organic acids such as acetic, lactic, propionic, benzoic, and sorbic acids are used in the food industry to control spoilage organisms. Benzoates, sorbates and propionic acid are primarily used as anti-fungal agents (Davidson, 2001). Natamycin, also known as pimaricin, an antibiotic agent produced by actinomycetae bacterium *Streptomyces natalensis*, is very effective against yeast and mould, and often used as anti-fungal surface application in foods (Davidson, 2001).

Microorganisms including yeast and mould are becoming resistant to preservatives (benzoates and sorbates) and antibiotics (natamycin) (Loureiro, 2000; Viljoen, 2001; Sanglard, 2002). A number of *Penicillium, Saccharomyces* and *Zygosaccharomyces* spp. can grow in the presence of potassium sorbate and degrade

it (Davidson, 2001). *P. roqueforti* has been found to be resistant to benzoate (Nielson and Dboer, 2000). The mould *Penicillium discolor* is found to have acquired resistance to natamycin even at a very high concentration (Filtenborg *et al.*, 1996; Nielson and Dboer, 2000). Thus alternative means are needed to control these organisms.

LAB are known to produce anti-microbial substances mainly in the form of organic acids and bacteriocins. Very few reports have been published about the production of specific anti-fungal substances from LAB especially from the probiotic bacteria.

El-Gendy *et al.* (1981) reported that a strain of *L. casei* inhibited growth and aflatoxin production of *Aspergillus parasiticus*. Suzuki *et al.* (1991) have reported anti-fungal activity of *Leuconostoc mesenteroids* strain from cheese. Bread spoilage moulds such as *Fusarium*, *Penicillium*, and *Aspergillus* are found to be inhibited by *L. sanfrancisco* CBI, an isolate from sourdough (Magnusson and Schnürer 2001).

Vandenberg (1993) reported production of a proteinaceous anti-fungal agent by *L. casei* subsp. *rhamnosus*. Anti-fungal peptides produced by *L. coryneformis* subsp. *coryneformis* (Magnusson and Schnurer, 2001) and *L. pentosus* (Okkers *et al.*, 1999) also are reported.

Rocken (1996) attributed the production of acetic acid to the anti-fungal activity observed in sourdough. Lavermicocca *et al.* (2000) found that this effect was due to phenyllactic acid and 4-hydroxyphenyllactic acid produced by *L. plantarum* together with lactic and acetic acids. This bacterium also found to produce anti-fungal low molecular weight substances such as benzoic acid, methylhydantoin, mevalonolacton, and anti-fungal cyclic peptides (Niku-Paavola *et al.*, 1999; Ström *et al.*, 2002). Fungi-static bacteriocin like substance pentocin TV35b was isolated from *L. pentosus* strain (Okkers *et al.*, 1999).

Short chain fatty acids in particular caproic acid produced by *L. sanfrancisco* CBI and found to be the inhibitory substances on sourdough bread spoilage molds such as *Fusarium*, *Penicillium*, and *Aspergillus* spp. (Magnusson and Schnürer, 2001).

The most common fermentation products that show anti-microbial activity are lactic, acetic and propionic acids (Bolm and Mortvedt, 1991). Acetic acid is the strongest inhibitor and has a wide range of inhibitory activity inhibiting yeast, mould

and bacteria (Bolm and Mortwedt, 1991). A mixture of lactic and acetic acid can effectively reduce the growth of *Salmonella typhimurium* more than other acid alone (Rubin, 1978).

The other main anti-microbial product of bacteria, the bacteriocin, is defined as ribosomally produced precursor poly-peptides or proteins that in their mature (active) form, exert an anti-microbial effect against a narrow spectrum of closely related bacteria (Jack *et al.*, 1995). While most bacteriocins produced by LAB have a narrow antibacterial spectrum, others are active against closely related species and *Listeria* and other food-borne pathogenic and spoilage organisms (Stiles, 1996; Grant and Salminen, 1998). Various organisms belonging to genera such as *Lactococcus, Pediococcus, Lactobacillus, Leuconostoc, Carnobacterium, Propionibacterium, Enterococcus, Bacillus* and *Escherichia* produce bacteriocins or bacteriocin-like inhibitory substances (Hoover and Steenson, 1993; Brandy-Smith, 1992; Klaenhammer, 1993).

Jenseniin G, a bacteriocin produced by *P. jensenii* P126, has narrow activity while propionicin PLG-1 produced by *P. thoenii* P127 inhibits propionibacteria, some fungi and *Campylobacter jejuni* (Lyon and Glatz, 1991). Nisin, a product of *Lactococcus lactis* spp. *lactis* inhibits *Lactococcus, Streptococcus, Staphylococcus, Micrococcus, Pediococcus, Lactobacillus, Listeria* and *Clostridia* (Klaenhammer, 1993; Jack *et al.*, 1994). Bacteriocin activity has been demonstrated in commercial preparations of *P. freudenreichii* culture supernatant that inhibits Gram negative bacteria (Al-Zoreky *et al.*, 1993). *P. jensenii* P126 inhibits the growth of certain strains of *Propionibacterium* spp., *Lactococcus* spp., and *Lactobacillus* spp. (Grinstead and Barefoot, 1992). Most bacteriocins are hydrophobic hence, they can be bound to fats and phospho-lipids. Nisin activity against *L. monocytogenes* is decreased in the presence of increasing fat concentration (Jung *et al.*, 1992). Jung *et al.* (1992) further indicated that, the inactivation by fat decreased with addition of nonionic emulsifiers such as Tween 80, but not by an anionic emulsifier such as lecithin.

The inhibitory effect of *L. bavaricus* was found to be greater in products stored at 4°C than at 10°C. Addition of 0.5% glucose has been reported to enhance the inhibitory effects of probiotics (Stiles, 1996). *Pediococcus acidilactici* produced the bacteriocin pediocin PA-1/ AcH (Marugg *et al.*, 1992). Diacetyl produced by LAB

is more anti-microbial against Gram negative bacteria and yeast and mould than Gram positive bacteria. LAB are the least affected (Jay, 1982). Jay (1982) also reported that the presence of glucose, acetic acid and Tween 80 reduced the anti-microbial activity of diacetyl. Better understanding of these antagonists may lead to targeted bio-control of spoilage flora and food-borne pathogens (Barefoot, 1993).

Except for the well-known pathogens, such as *Salmonella,* the presence of pathogens in ready-to-eat foods like dips has received little attention in the past. The bacterium *L. monocytogenes* has become a major concern in the food industry in recent years. High mortality rate, particularly among the very young and the old and the infirm has been the primary reason for the concern of listeria infections (Schwartz *et al.*, 1988). Moreover, the bacterium is widespread in nature and exceedingly difficult to keep out of food, particularly in unprocessed foods (Brackett, 1988). Another deadly food pathogen that causes haemorrhagic colitis, *Escherichia coli* 0157:H7 has also been reported to grow on foods stored at refrigeration temperatures (Palumbo, 1987; Prescott, 1999). Not only the growth of the pathogenic bacteria but also yeast and mould at refrigeration temperature is also an enormous problem in the fast food industry mainly with the fresh foods where addition of artificial preservatives is not accepted by the consumers. The inhibitory substances produced by LAB, bifidobacteria and propionibacteria can help in overcoming this hazard.

2.5.3 Technological aspects of probiotics

To exhibit health benefits to the host, probiotic organisms should be provided with conditions in which they survive in food and be carried to the functional site in the human body in functional numbers. Consumers purchase products with probiotics with the assumption that large numbers of probiotic organisms are viable in the product when consumed. In many instances, the number of viable probiotic organisms during storage of the product or at the time of purchase of the product are not being evaluated. It is important to evaluate how far the food products match and retain during storage, the conditions required by probiotics.

There are many examples available for positive inter-species interactions of probiotics. Cheng and Nagasawa (1983) found that the growth of bifidobacteria was greatly stimulated when inoculated along with *L. casei* in milk. Kaneko *et al.* (1994)

indicated that a bifidogenic factor produced by *Propionibacterium freudenreichii* enhanced the growth of *Bifidobacterium longum, B. bifidum, B. adolescentis, and B. breve.* Shimamura *et al.* (1992) reported that *B. infantis, B. breve, B. longum* were less sensitive to oxygen present. Incorporation of *S. thermophilus* reduces the oxygen content (Ishibashi and Shimamura, 1993) thereby favouring the growth of *B. infantis, B. breve* and *B. longum. L. acidophilus* and *B. bifidum* grew well at low surface tension and were resistant to lysozymes (Gilliland, 1978). Synergistic growth promoting effects between *L. acidophilus* and *B. bifidum* are known to occur (Kneifel *et al.,* 1993). The growth rate of *L. acidophilus* was not affected by *B. bifidum,* but the latter organism was repressed unless the initial inoculum was in the ratio of 10^4: 10^3 *(B. bifidum: L. acidophilus)* (Rasic and Kurmann, 1983).

Babu *et al.* (1992) indicated that tomato juice and papaya pulp stimulated the growth of *L. acidophilus* and resulted in higher viable counts, shorter generation time and improved sugar utilisation. Ahmed and Mital (1990) suggested that growth promoters of *L. acidophilus,* such as magnesium and manganese in these products may stimulate the growth of *L. acidophilus.* The survival of *B. longum* in milk has been shown to improve by adding 0.01% baker's yeast (Shimamura, 1982). Chaia *et al.* (1998) observed a competitive inhibition of *P. acidipropionici* by mixed culturing with *Lactobacillus helveticus.* Propionibacteria are a slow grower org. compared to LAB.

The survival of probiotics through the intestinal tract is strain-specific (Tamime *et al.,* 1995). Therefore, selecting probiotic strains that survive well in the intestine is the prime technical challenge. Secondly, the selected strains should have a good survival rate until it is consumed. The Fermented Milks and Lactic Acid Beverages Associations of Japan has developed a standard which requires a minimum of 10^7 viable bifidobacteria cells per millilitre to be present in fresh dairy products (Ishibashi and Shimamura, 1993). National Yoghurt Association (NYA) of the United States specifies 10^8 cfu g^{-1} lactic acid bacteria at the time of manufacture (Roberts and Maust, 1995).

Many factors can affect the viability of probiotic microorganism in the carrier food including the strains used, interaction between species present, culture conditions, chemical composition of the food, final acidity, milk solid content, availability of nutrients, growth promoters and inhibitors, concentration of sugars

and salt (osmotic pressure), dissolved oxygen (especially for bifidobacteria), level of inoculation, incubation temperature, fermentation time and storage temperature (Hamann and Marth, 1983; Costello, 1993; Bertoni *et al.,* 1994; Young and Nelson 1978; Kneifel *et al.*, 1993; Lankaputhra and Shah, 1995; Dave and Shah, 1997; Rybka, 1994).

2.6. Gums as food additives

Xanthan gum is a high molecular weight anionic polysaccharide. It is an exo-cellular polysaccharide produced by fermentation of the bacteria *Xanthomonas campestris.* It is used to improve rheology of foods in aqueous systems and as a stabilizer for emulsions and suspensions. Its numerous areas of applications cover a broad spectrum and range in the food industry. Major functions of xanthan gum in food products include stabilization of emulsions, inhibition of syneresis, provision of good cling, improvement of texture, imparting a creamy consistency to the product, enhancing mouthfeel, contributing body to the product, imparting viscosity, stabilizing insoluble ingredients, stabilizing pulp in beverages, providing emulsion and foam stability, controlling sedimentation, controlling drift and cling, providing fine pore structure, controlling rheology, providing temperature and pH stability and binding water. Xanthan gum is used in dressings and mayonnaise, sauces, ketchup, soups, desserts, bakery fillings and cake mixes, dairy (yoghurt, yoghurt milk shakes, creamed cottage cheese) and fruit juices and fruit preparations (Araujo, 1967; Dintzis *et al.*, 1970; Fennema, 1996; Rhodia Food, 2002).

2.7. Selective enumeration of yoghurt bacteria and probiotic bacteria

An important parameter in monitoring the quality of food products is the ability to estimate the claimed presence/number of probiotic bacteria such as *L. acidophilus, Bifidobacterium, L. casei* and *L. rhamnosus,* differentially. Several selective media have been developed for the enumeration of yoghurt cultures, *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus.* These include, lactic acid bacteria agar (Davis *et al.*, 1971), Lee's agar (Lee *et al.,* 1974), acidified reinforced clostridial agar (Johns *et al* 1978), acidified MRS agar (Dave and Shah, 1996) and M17 agar (Jordono *et al.,* 1992). *S. thermophilus* agar was used in the selective enumeration of *S. thermophilus* (Dave and Shah, 1996; Shah, 1999).

Several media have been recommended for the selective enumeration of *L. acidophilus,* including bile medium (Collins, 1978), Rogosa agar, DeMan Rogosa Sharpe (MRS) medium containing maltose, rafinose or melibiose in place of dextrose (Hull and Roberts, 1984), Cellobiose-esculin agar (Hunger, 1986) and agar medium based on X-Glu (Kneifel and Pacher, 1993). Similarly, several selective media have been developed for the enumeration of pure cultures of bifidobacteria including nalidixic acid- neomycin sulfate-lithium chloride- paromomycin sulfate agar (Laroia and Martin, 1991). Several other media were suggested by others (Burford, 1989; Munoa and Pares, 1988; Onggo and Fleet, 1993; Samona and Robinson, 1994; Sozzi *et al.*, 1990). However, these media are not suitable for selective enumeration of *L. acidophilus* and bifidobacteria in the presence of yoghurt culture organisms or other bacteria such as *L. casei, L. reuteri* and *L. plantarum.* In addition to this, differences exist among the strains of the same bacterial species in the tolerance to low pH, bile salts, NaCl and in sugar fermentation characteristics (Kim, 1988). Selective enumeration of *L. casei* in fermented milk products such as yoghurt containing probiotic bacteria based on 15° C incubation temperature and 14-day incubation time was suggested by Champagne *et al.* (1997). However, a 14-day incubation period may not be practical for the dairy industry if the results are required in a short time. *L. casei* (LC) agar has been developed by Ravula and Shah (1998b) to selectively enumerate *L. casei,* but in situations where propionibacteria are present, this media cannot be used. To selectively enumerate propionibacteria, sodium lactate agar was suggested in the Handbook of Microbiological Media (Atlas and Parks, 2000). This media again may not be suitable in the presence of other culture bacteria. While the current trend is to use a consortium of probiotic bacteria in a food product, it is important to identify suitable media to enumerate the number of viable cells of individual bacteria in a consortium of bacteria.

CHAPTER 3

SELECTIVE ENUMERATION OF *LACTOBACILLUS DELBRUECKII* SUBSP. *BULGARICUS*, *STREPTOCOCCUS THERMOPHILUS*, *LACTOBACILLUS ACIDOPHILUS*, *BIFIDOBACTERIUM* SPP, *LACTOBACILLUS CASEI* AND *LACTOBACILLUS RHAMNOSUSM* AND PROPIONIBACTERIA

3.1. Introduction

A number of health benefits have been claimed for probiotic bacteria and more than 90 probiotic products containing one or more groups of probiotic organisms are available worldwide. Probiotic food can be defined as "food containing live microorganisms which to actively enhance the health of consumer by improving the balance of microflora in the gut" (Fuller, 1992).

A number of probiotic organisms including *L. acidophilus*, *Bifidobacterium* spp. *Lactobacillus casei*, *Lactobacillus rhamnosus*, and *Propionibacterium* are incorporated in dairy foods. These organisms grow slowly in milk during product manufacture. Therefore the usual practice is to incorporate yogurt bacteria (*Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*) along with probiotic cultures. Yoghurt bacteria do not survive in the gastric passage or colonize in the gut (Shah and Jelen, 1990) and are unlikely to provide any therapeutic benefits. However, yoghurt bacteria grow rapidly and thus are added to speed up the fermentation process.

To provide health benefits, the suggested concentration for probiotic bacteria is 10^6 cfu g^{-1} of a product (Shah, 2000). It seems reasonable to assume that the beneficial effects of probiotic bacteria can be expected only when viable cells are ingested. An important parameter in monitoring viable organisms in assessing product quality is the ability to count probiotic bacteria differentially. Differential enumeration of probiotic bacteria is difficult owing to the presence of several types of similar microbes in a product. In order to assess viability and survival of probiotic bacteria, it is important to have a working method for selective enumeration of these bacteria.

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Several media for selective enumeration of *L. acidophilus* and *Bifidobacterium* spp. have been previously proposed (Hunger, 1986; Hull and Roberts, 1984; Wijsman et al., 1989; Laroia and Martin, 1991; Dave and Shah, 1996; Lankaputhra et al., 1996; Shah, 1997, 2000). Similarly, several media have been proposed for selective enumeration of yogurt cultures (Onggo and Fleet, 1993; Samona and Robinson, 1984). There are only few reports that have described selective enumeration of *Lactobacillus casei* in the presence of other probiotic bacteria and yogurt bacteria (Champagne *et al.*, 1997; Ravula and Shah, 1998). Selective enumeration of *L. casei* in probiotic products based on a 15°C incubation temperature and 14 day incubation time was studied by Champagne *et al.* (1997). Ravula and Shah (1998) developed a medium known as LC agar for selective enumeration of *L. casei*. Selective enumeration of *Lactobacillus reuteri*, *Lactobacillus plantarum*, *Lactobacillus rhamnosus*, and propionobacteria has not been studied extensively. The aim of this study was to develop media for selective enumeration of *S. thermophilus*. *L. delbrueckii* ssp *bulgaricus L. acidophilus L. casei*, *L. rhamnosus,* bifidobacteria and propionibacteria.

3.2. Materials and methods

3.2.1. Bacteria Cultures and Propagation

L. delbrueckii ssp *bulgaricus* (LB 100), S. *thermophilus* (ST 2362), *L. casei* (DS 930), *L. acidophilus*, (DS 910), *Bifidobacterium lactis* (DS920), and *Propionibacterium fredenreichii* subsp. *shermanii* and *Propionibacterium fredenreichii* subsp. *globosum* (Type standard 10360) were provided by DSM Gist brocades (DSM Gist brocade Australia Pty Ltd., Werribee, Australia). *L. paracasei* subsp. *paracasei* (LC01), *L. acidophilus* (LA 5), *Bifidobacterium lactis* (Bb 12), and *Propionibacterium fredenreichii* subsp. *shermanii* (PS1) were obtained from Chr. Hansen (Chr. Hansen Pty. Ltd., Bayswater, Australia). *L. rhamnosus* (LC 705), *L. acidophilus* (74-2), *Bifidobacterium* spp. (BB 420), were received from Danisco Cultor (Danisco Cultor, Dingley, Australia). S. *thermophilus* (TA040) *L. paracasei* subsp. *paracasei* (LBC81), *L. rhamnosus* (LBA), *L. acidophilus* (LAC 4), and *Bifidobacterium* spp. (BL), were from Rhodia (Rhodia Australia Pty. Ltd., Notting Hill, Australia).

All the strains were tested for purity using Gram stain. All the cultures except propionibacteria were propagated weekly in sterile 12% reconstituted skim

milk (RSM) supplemented with 2% glucose and 1.2% yeast extract. The cultures were grown using 1% inoculum for 18 hours at 37°C. Propionibacteria were grown in sodium lactate broth (composition; 10g of pancreatic digest of casein, 10g of sodium lactate, 10g of yeast extract, 0.5g of Tween 80, and distilled water 1 L) and incubated at 30°C for 2 days using 1% inoculum. Bifidobacteria were propagated using 1% inoculum in sterile RSM supplemented with 0.05% L-cysteine·hydrochloride in order to provide anaerobic condition and to enhance their growth. The cultures were maintained in the same media at 4° C. Before enumeration the cultures were transferred successively three times.

3.2.2. Media preparation

Bacteriological peptone and water diluent Bacteriological peptone and water diluent (0.15%) was prepared by dissolving 1.5 g of bacteriological peptone (Oxoid Australia Pty Ltd., West Heidleberg, Australia) in 1 L of distilled water. The pH was adjusted to 7.0 ± 0.2, followed by autoclaving 9 mL aliquots at 121°C for 15 min.

3.2.2.1. Streptococcus thermophilus agar (ST agar)

The ST agar was prepared according to the method described by Dave and Shah (1996).

3.2.2.2. MRS agar, pH-modified (pH 5.2, 4.58) MRS agar, MRS-bile (0.2% and 0.5%) agar, MRS-NaCl agar, and MRS-LiCl agar Rehydrated MRS broth (Oxoid) was prepared according to the manufacturer instructions. The pH of the broth was adjusted to 5.2 and 4.58 using 1.0 M HCl to obtain the pH-modified agar. Two and five grams of pure bile salts (Amyl Media, Dandenong Australia)/ L were added to obtain 0.2% and 0.5% MRS-bile agar. Forty grams of NaCl / L was added for MRS-NaCl agar (4% final concentration) and 5 g /L lithium chloride (LiCl) was added for MRS-LiCl agar (0.5% final concentration). Agar powder was added to each broth at the rate of 1.2% and the media were autoclaved at 121°C for 15 min. Inoculated plates in duplicates were incubated anaerobically at 37°C and 43°C for 72 h.

3.2.2.3. *MRS-vancomycine agar*

The MRS-vancomycine agar was prepared by adding 2 mL of filter sterilized, 0.05 g vancomycine (Sigma Chemical Co., Castle Hill, Australia) /100 mL solution to 1 L of autoclaved MRS agar just before plating, to obtain 1 mg/L final concentration.

3.2.2.4. MRS-NNLP agar

The MRS-NNLP (nalidixic acid, neomycine sulfate, lithium chloride and paramomycine sulfate; Sigma Chemical Co., Castle Hill, Australia) agar was prepared according to the method described by Laroia and Martin (1991). MRS agar was the basal medium. Filter sterilized NNLP was added to the autoclaved MRS base just before pouring. Filter sterilized L-cysteine·HCl (0.05% final concentration) was also added at the same time to lower the oxidation-reduction potential of the medium and to enhance the growth of anaerobic bifidobacteria. Inoculated plates in duplicates were incubated at 37°C anaerobically for 72 h.

3.2.2.5. Reinforced clostridial agar (RCA)

RCA agar (Oxoid) was made according to the manufacturer instructions and sterilized by autoclaving at 121°C for 15 min.

3.2.2.6. Basal agar (BA), BA-maltose, BA-galactose, BA-sorbitol, BA-mannitol, BA-esculin agar

Basal agar (BA) was prepared (composition: 10 g of trypton, 10g of Lablemco powder, 5 g of yeast extract, 1 g Tween 80, 2.6 g of K_2HPO_4, 5 g of sodium acetate, 2 g of tri-ammonium citrate, 0.2 g of $MgSO_4.7H_2O$, 0.05 g of $MnSO_4.4H_2O$, 12 g of bacteriological agar, and 1 L of distilled water) and autoclaved at 121°C for 15 min. Ten milliliters of membrane filtered sterile 20% solutions of maltose, galactose, sorbitol, manitol or esculin were added to 90 mL of basal agar (2% final concentration) just before pouring the agar medium. Inoculated plates in duplicates were incubated aerobically and anaerobically at 37°C and 43°C for 72 h.

3.2.2.7. Sodium lactate agar (NaLa agar), Arabinose agar, Xylose agar, Raffinose agar.

The base for these agar media were prepared(composition: 10 g of pancreatic digest of casein, 10g of Sodium lactate, 10g of yeast extract, 2g of sodium pyruvate, 2g of glycine,1.5 g of sodium chloride, 0.5g of Tween 80, 0.25 g of di-potasium hydrogen phosphate, 12 g of bacteriological agar and 1 L of distilled water). The pH was adjusted to 7 $\pm$ 0.2 using 1M HCl and 10M NaOH. To make NaLa agar, 10 g of sodium lactate was added before autoclaving. The mixture then was autoclaved at 121°C for 15 min. For other agar media, 10mL of 10% membrane filtered arabinose, raffinose, or xylose, were added to 90 mL of autoclaved media before pouring the plate. Inoculated plates in duplicates were incubated at 30°C anaerobically for 7-9 days.

3.2.2.8. LC agar

LC agar was made using the method described by Ravula and shah (1998). The incubation was carried out under anaerobic condition at 27 °C for 72h

3.2.3. Enumeration of bacteria

Cultures were activated by three successive transfers in a nutrient medium (reconstituted skim milk for probioting bacteria and NaLa-broth for *Propionibacterium*) before enumeration. One gram of each culture was 10-fold serially diluted (10^3 to 10^7) in 0.15% sterile bacteriological peptone and water diluents. The enumeration was carried out using the poure plate technique. Anaerobic jars and gas generating kits (Anaerobic System BR 38; Oxoid Ltd., Hampshire, England) were used for creating anaerobic condition Plates containing 25 to 250 colonies were enumerated and recorded as colony forming units (CFU) per gram of the product or culture.

All the experiments and analyses were repeated at least twice. The results presented are averages of two replicates.

3.3. Results and discussion

3.3.1. Evaluation of enumeration methods

Viable counts (in log $_{10}$) and colony size (in mm diameter) of 7 species of bacterial cultures containing 18 strains of bacteria including 1 strain of *L. delbrueckii* subsp. *bulgaricus*, 2 strains of *S. thermophilus*, 3 strains of *L. casei*, 2 strains of *L. rhamnosus* 4 strains of *L. acidophilus*, 4 strains of *Bifidobacterium* spp. and 2 strains of propioni bacteria in basal agar with various sugar media are presented in Table 3.1. *L. delbrueckii* subsp. *bulgaricus* did not grow in any sugar-based media except in MRS agar. MRS agar is particularly suitable for growing lactobacilli.

RCA agar supported the growth of all tested organisms. Bifidobacteria grew in this medium even without the addition of L-cysteine hydrochloride (data not shown). Therefore RCA agar is not suitable for selective enumeration.

ST agar was found to be suitable for *S. thermophilus* (data not shown). *S. thermophilus* formed tiny (0.1-0.5 mm) colonies in ST agar at 37°C under aerobic incubation after 24 h. The incubation time was insufficient for growth of other cultures even if ST agar did not inhibit the growth of other organisms. Therefore ST agar at 37°C for 24 h and aerobic condition were selective for *S. thermophilus*. This is in agreement with previous reports (Dave and Shah, 1996).

Other organisms such as, *L. casei*, *L. rhamnosus* and *L. acidophilus* grew in all sugar based media. *Bifidobacterium* did not grow in any media, except in BA-esculin agar when L cystein hydrocloride was not present in the media. *Propionibacterium fredenreichii* subsp. *Shermanii* grew in MRS and BA-galactose agar only and the colony size were much smaller than others. Thus, probiotic organisms could not be selectively enumerated based solely on sugar utilization pattern. Table 3.2 shows the counts of bacterial cultures in media containing different inhibitory substances including vancomycine, NNLP, hydrochloric acid, NaCl, LiCl, and bile at 37°C or 43°C incubations. All the organisms except *Bifidobacterium* spp. grew in MRS agar. When the pH of MRS agar was reduced to 5.2 and the incubation temperature increased to 43°C, only *L. delbrueckii* subsp. *bulgaricus* (which formed 1.0 mm, white rough irregular colony), *L. rhamnosus* (which formed 2 mm, shiny smooth white colony) and *L. acidophilus* (which formed 0.1-0.5 mm brown rough irregular colonies) showed good growth. When the pH of MRS agar was reduced to 4.58 using 1M HCl, only *L. delbrueckii* subsp. *bulgaricus* and *L. rhamnosus* showed good growth similar to that formed in -MRS agar at pH 5.2 and the growth of *L. acidophilus* was inhibited except that of DS 910. Therefore

MRS agar at pH 4.58, under anaerobic incubation at 43°C could be selective for *L. delbrueckii* subsp. *bulgaricus* if *L. rhamnosus* and *L. acidophilus* DS 910 are not present in a product. The colony morphology of *L. delbrueckii* subsp. *bulgaricus* and *L. rhamnosus* was very different and these two organisms can be easily differentiated if *L. rhamnosus* was present in the product. Therefore, pH modified agar MRS (pH 4.58) under anaerobic incubation at 43°C can be used to selectively enumerate *L. delbrueckii* subsp. *bulgaricus* from a product

L. casei grew in MRS- NaCl (4%), MRS-LiCl (0.5%) at 37°C under anaerobic incubation and in LC agar. *L. casei* did not grow in NNLP agar and at 43°C. Lower incubation temperatures (≤37°C) supported the growth of *L. casei. L. casei* and *L. rhamnosus* were resistant to 1mg vancomycine/L. *L. casei* and *L. rhamnosus* formed well developed smooth white discs like colonies that were 2 mm or more in diameter. *L. rhamnosus* grew at both incubation temperatures of 37°C and 43°C and in all sugar based media under aerobic and anaerobic conditions except in MRS-NNLP agar and showed varying growth pattern (between strains) in MRS-bile agar, MRS- NaCl agar and in MRS-LiCl agar. The organisms grew well in MRS- V agar at incubation temperatures of 37°C and 43°C and in LC agar at 27 °C. MRS-V agar at 43°C anaerobic incubation supported the growth of only *L. rhamnosus.* No other cultures tested including, *L. delbrueckii* subsp. *bulgaricus, S. thermophilus*, *L. casei*, *L. acidophilus*, *Bifidobacterium lactis* and *Propionibacterium* grew in this medium.

MRS-V agar at 37°C or LC agar at 27°C under anaerobic incubation (Table 3.2) could be selective for *L. casei* when *L. rhamnosus* was not present in a product. When *L. rhamnosus* was present, total count of *L. casei* and *L. rhamnosus* could be obtained using MRS-V agar at 37°C and anaerobic incubation for 72h. The count of *L. rhamnosus* on MRS-V agar at 43°C under anaerobic incubation 72h could be subtracted from the total count of *L. casei* and *L. rhamnosus* to obtain the count of *L. casei.*

MRS- NNLP agar (which contains 0.05% L-cysteinein the formula) at 37°C anaerobic incubation supported the growth of only bifidobacteria (Table 3.2). But when L-cysteine is not present in the media, bifidobacteria either did not grow or formed pinpoint colonies (data not shown). Therefore, MRS- NNLP agar with 0.05% L-cysteine, anaerobic incubation at 37°C were selective for bifidobacteria and

the absence of L-cysteine was able to control the growth of bifidobacteria from other media.

Table 3.3 shows the colony counts and colony sizes of various bacterial cultures in different agar media. Colonies of ≥0.5 mm diameter only counted as developed colonies for the enumeration purpose. NaLa agar, arabinose agar, raffinose agar and xylose agar supported the growth of *L. casei L. acidophilus* and *L. rhamnosus* as well as of propionibacteria. In these media *L. casei* and *L. rhamnosus* formed white shiny smooth colonies of 1mm diameter. Propionibacteria formed colonies of 0.5 mm diameter in all the media. However, in NaLa agar propionibacteria formed colonies that were dull brown with lighter margin of 1.00mm-2.5mm in diameter. The colonies were very different to those formed by *L. casei* and *L. rhamnosus*. *L. acidophilus* formed pinpoint colonies. To eliminate the possibility of *L. acidophilus* being counted, colonies of ≥0.5mm diameter were only counted. Proper colonies of Propionibacteria formed only after 72 hours of incubation and after 7 days colony size grew to 2 mm diameter. The colony sizes of *L. casei* and *L. rhamnosus* did not change. In NaLa agar the recovery rate was the highest and colony morphology and colony size were different than those formed in arabinose agar, and xylose agar. Thus, NaLa agar could be used to selectively enumerate propionibacteria. The propionibacteria could also be counted by subtracting the counts of *L. casei* and *L. rhamnosus* on day 3 form the total count of *L. casei, L. rhamnosus* and propionibacteria obtained on day 7 (Table 3.3).

BA-sorbitol agar and BA-manitol agar at 43°C aerobic incubation (Table 3.4) and BA-sorbitol agar and BA-manitol agar at 43°C anaerobic incubation (data not shown) also supported only *L. rhamnosus*. Therefore MRS-V agar at 43°C under anaerobic incubation, BA-sorbitol agar or BA-manitol agar at 43°C either under aerobic or anaerobic incubations are selective for *L. rhamnosus*.

L. acidophilus was found to be the most difficult to selectively enumerate, since most of the media that supported the growth of *L. acidophilus* also supported the growth of *L. casei* and *L. rhamnosus*. When the incubation temperature was increased to 43°C, *L. casei* was eliminated. But *L. rhamnosus* still formed well-developed (1.5 mm in diameter) colonies and *L. acidophilus* formed smaller colonies (0.1-1.0mm) depending on the sugar used (Table 3.1). When galactose was used, bifidobacteria formed pin-point colonies in the absence of L- cysteine, and the

colonies could be confused with *L. acidophilus*. *L. delbrueckii* ssp *bulgaricus* and *S.* thermophilus formed colonies in MRS agar when incubated anaerobically at 43°C. Therefore, MRS agar anaerobic incubation at 43°C can be used to enumerate *L. acidophilus* when *L. delbrueckii* subsp. *bulgaricus* and *S.* thermophilus were not present in the product (data not shown).

When incubated anaerobically at 43°C in BA-manitol, BA-sorbitol, BA-esculin and BA-maltose agar, *L. rhamnosus* formed large (2.00-2.5 mm diameter) smooth shiny disc like colonies, (data not shown) while *L. acidophilus* formed smaller (0.1-1.0 mm diameter) rough dull colonies. BA-maltose agar supported the growth of *L. acidophilus* more than the other BA based agar media at this incubation temperature, but one strain of *L. acidophilus* DS 910 formed larger colonies that could be confused with *L. rhamnosus*. In MRS agar, *L. delbrueckii* ssp *bulgaricus L. rhamnosus* and *L. acidophilus*, formed colonies (Table 3.1) while in BA maltose agar only *L. acidophilus* and *L. rhamnosus* formed colonies. *L. rhamnosus* formed larger (2.00-2.5 mm diameter) smooth, shiny, disc like colonies, while strains of *L. acidophilus* formed smaller rough brownish colonies of 0.1-1.0 mm diameter that could be easily distinguished. Therefore, MRS agar under aerobic or anaerobic incubation at 43°C could be used to count *L. acidophilus* when *L. delbrueckii* subsp. *bulgaricus* is not in the product. When *L. delbrueckii* subsp. *bulgaricus* is present, BA-maltose agar under anaerobic incubation at 43°C can be used and only small rough brownish colonies should be counted as *L. acidophilus*.

Among the media tested for *L. acidophilus*, BA-sorbitol agar gave the highest recovery rate (Table 3.1). In this medium, *L. casei* and *L rhamnosus* formed shiny big smooth white colonies while all strains of *L. acidophilus* tested formed rough dull small brownish colonies. Therefore, only the small dull rough brownish colonies should be counted as the count of *L. acidophilus*.

Table 3.5 summarizes the media that could be used for selective enumeration of seven groups of bacteria and their incubation conditions and colony morphology. To verify the efficacy of the method selected in this study, mixtures of *L. delbrueckiisubsp. Bulgaricus, S. thermophilus, L. acidophilus,* bifidobacteria, *L. casei, L. rhamnosus* and propionibacteria cells were added at approximately 10^7 cfu/mL in the ratio of 0.1, 0.5, 4, 2, 1, 1 and 1, respectively, and the organisms were plated in the media under incubations outlined in Table 3.5. As shown in the table,

the media were discriminatory for the various groups of bacteria. Thus it appears that the methods could be used for selective enumeration of the seven groups of the seven groups of bacteria used in this study.

3.3.2. Enumeration of bacteria in Commercial products

Because the evaluation of media for selective enumeration of yoghurt and probiotic bacteria was carried out using pure cultures, it was desirable to validate the efficacy of the method selected using commercial products. Five brands of commercial yoghurts (names not disclossed) and one brand of Swiss cheese (name not disclossed) were purchased from local supper market and their bacterial populations analyzed using the different selective bacteriological media. Enumeration of *S. thermophilus* was carried out using ST agar aerobic incubation at 37°C 24h. *L. delbrueckii* ssp *bulgaricus* was enumerated on MRS-agar (pH 4.58) anaerobic incubation at 45°C for 72h. For *L. rhamnosus* MRS-V agar anaerobic incubation at 43°C was used. *L. casei* was enumerated using subtraction method, where viable count of *L rhamnosus* on MRS-V agar at 43°C under anaerobic incubation was subtracted from the total count of *L. casei* and *L rhamnosus* on MRS-V at 37°C under anaerobic incubation. Bifidobacteria were enumerated on MRS-NNLP agar. Enumeration of *L. acidophilus* was carried out in BA-sorbitol agar at 37°C under anaerobic incubation, and BA-maltose 43°C anaerobic incubation for 72h, where only the small rough brownish colonies (0.1-0.5mm) were counted as *L. acidophilus*. Propionibacteria were enumerated using subtraction method, where the day 3 count of lactic acid bacteria on NaLa agar, 30°C anaerobic incubation was subtracted from the day 7 total count of lactic acid bacteria. and propionibacteria.

Table 3.6 shows the organisms claimed to be present and the actual recovery of the organisms. *S. thermophilus* was present in all of yoghurts tested. *L. delbrueckii* subsp. *bulgaricus* was present only in product 5 (sknny yoghurt). Many commercial products are manufactured using *L. acidophilus*, Bifidobacteria and *S. thermophilus* (ABT) cultures, which do not contain *L. delbrueckii* subsp. *Bulgaricus*. *L. casei* was claimed to be present in both products 4 and 5, however, only product 4 (natural yoghurt) showed reasonable population of this organism. The stage of shelf life and the pH of yoghurt might have affected the viability of the probiotic organism.

Product 2 (natural yoghurt) and product 3 (flavoured yoghurt) had high counts of all organisms claimed including *S. thermophilus*, *L rhamnosus*, *L. acidophilus*, and bifidobacteria. Products 2 and 3 contained *L rhamnosus*. Bifidobacteria were found in all products 4 and5 and products 2 and 3 in high concentrations (10^6- 10^7). *L. acidophilus* also was found in appreciable concentration in all yoghurt claimed to contain this organism. Propionibacteria were found only in Swiss cheese (product 6) and that was the only product claimed to contain propionibacteria. The identity of the organisms was confirmed using gram stain. Thus it appears that the enumeration methods developed and selected in this study were suitable for enumeration of *S. thermophilus*, *L. delbrueckii* ssp *bulgaricus*, *L. casei*, *L rhamnosus*, *L. acidophilus,* bifidobacteria and propionibacteria.

3.4. Conclusion

In this study, 19 bacteriological media were evaluated under different incubation conditions for their suitability to recover and enumerate 7 species containing 18 strains of bacteria including *S. thermophilus*, *L. delbrueckii* ssp *bulgaricus*, *L. casei*, *L rhamnosus*, *L. acidophilus,* bifidobacteria and propionibacteria. The evaluation was based on sugar fermentation patterns, resistance to inhibitory substances, (such as acid, bile, salts and antibiotics), different incubation temperatures (27°C, 30°C, 37°C 43°C and 45°C), incubation condition (such as aerobic and anaerobic) and the duration of incubation (24h, 72h, 7-9days). ST agar 37°C 24h under aerobic incubation is suitable for *S. thermophilus*. *L. delbrueckii* subsp. *bulgaricus* could be enumerated in MRS agar (pH 4.58 or pH 5.2) anaerobic incubation for 72h. MRS-vancomycine agar and anaerobic incubation at 43°C for 72h were selective for enumeration of *L rhamnosus*. BA- sorbitol agar or BA-manitol agar at 43°C and either aerobic or anaerobic incubation could be used for the enumeration of *L rhamnosus*. MRS-vancomycine agar and anaerobic incubation at 37°C, for 72h or LC agar at 27°C for 72h under anaerobic incubation were selective for enumeration of *L. casei*. *L. casei* could be enumerated by subtraction method when *L rhamnosus* was present in the product. The count of *L rhamnosus* on MRS- vancomycine agar under anaerobic incubation at 43°C for 72h could be subtracted from the total counts

of *L. casei* and *L rhamnosus* on MRS- vancomycine agar at37°C, for 72h under anaerobic incubation to obtain *L. casei* count. Bifidobacteria could be enumerated on MRS-NNLP agar. The most suitable method for counting propionibacteria was by subtracting the counts at day three of all bacteria except propionibacteria on NaLa agar under anaerobic incubation at 30°C Subtraction method of subtracting day 3 count of all cultures except propionibacteria on NaLa agar from the total counts at day seven of all bacteria including propionibacteria under same incubation conditions. Table 3.6 summerises that recommended media for selective enumeration of different bacteria. Counting large (1.0-2.5 mm diameter), smooth brownish colonies with lighter margin on sodium lactate agar after 7-9d at 30°C under anaerobic incubation could also be used to count propionibacteria. *L. acidophilus* could be enumerated on BA-sorbitol agar at 37°C for 72h under anaerobic incubation or in BA-maltose 43°C anaerobic incubation or on MRS agar at 43°C under anaerobic incubation.

Table.3.1 Viable counts ($\log_{10}$ cfu g^{-1}) and colony size of bacterial cultures in different sugar-based media (anaerobic incubation, 37°C 72h)

Cultures (Full names in footnots)	MRS		BA-maltose		BA-galactose		BA-sorbitol		BA-mannitol		BA-esculin	
	Counts	Size-(mm)	Counts	Size-(mm)	Count	Size (mm)	Counts	Size (mm)	Counts	Size (mm)	Counts	Size (mm)
LB- LB 100	8.3	0.1-0.5	<3.0	-	-	-	<3.0	-	<3.0	-	<3.0	-
ST- TA 040	7.1	0.5	<3.0	-	<3.0	-	<3.0	-	<3.0	-	<3.0	-
ST- DS 2362	8.1	0.5	<3.0	-	<3.0	-	<3.0	-	<3.0	-	<3.0	-
LC- DS 930	9.3	2.0	9.5	2.0	9.3	2.0	9.2	2.0	9.4	2.0	9.2	2.0
LC- LCO 1	9.3	2.0	8.3	2.0	9.2	2.0	9.2	2.0	9.2	2.0	9.2	1.0-1.5
LC- LBC 81	9.1	2.0	9.3	2.0	9.2	2.0	9.3	2.0	9.2	2.0	9.2	1.0-1.5
LR- LC 705	9.2	2.0	9.3	2.0	9.1	2.0	9.2	2.0	9.4	2.0	9.2	1.0-1.5
LR- LBA	9.4	2.0	9.4	2.0	9.0	2.0	9.4	2.0	9.4	2.0	9.1	1.0-1.5
LA- LA 5	7.0	0.1-0.5	8.0	0.5-1	7.7	0.5-1	8.1	0.1-0.5	7.4	0.1-0.5	7.5	0.1-0.5
LA- DS 910	8.4	0.5-1	8.6	1.0- 1.5	8.3	1.0-1.5	8.7	0.1-0.5	9.2	0.1-0.5	7.8	0.1-0.5
LA- LAC 4	7.2	0.1-0.5	7.1	0.5-1.0	7.2	.5-1	7.1	0.1-0.5	7.1	0.1-0.5	7.8	0.1-0.5
LA- 74-2	7.1	0.1-0.5	8.8	0.5-1.0	7.3	.5-1	8.1	0.1-0.5	7.2	0.1-0.5	7.2	0.1-0.5
BB- Bb 12	<3.0	-	<3.0	-	<3.0	-	<3.0	-	<3.0	-	9.3	1.0-1.5
BB- DS 920	<3.0	-	<3.0	-	<3.0	-	<3.0	-	<3.0	-	9.0	1.0-1.5
BB- 420	<3.0	-	<3.0	-	<3.0	-	<3.0	-	<3.0	-	8.9	1.0-1.5
BB- BL	<3.0	-	<3.0	-	<3.0	-	<3.0	-	<3.0	-	<3.0	-
PS- PS 1	10.6	0.1-0.5	<3.0	-	8.1	0.5-1.0	<3.0	-	<3.0	-	<3.0	-
PS- 10360	10.8	0.1-0.5	<3.0	-	8.9	0.5-1.0	<3.0	-	<3.0	-	<3.0	-

LB = *L. delbrueckii* subsp. *bulgaricus*, ST =*S. thermophilus*, LC = *L.* casei, LA = *L. acidophilus*,

LR= *L. rhamnosus*, BB = *Bifidobacterium*, PS = *Propionibacterium freudenreichii* subsp. *shermanii*.

Table.3.2 Viable counts ($\log_{10}$ cfu g^{-1}) of bacterial cultures under anaerobic incubation at 37°Cand 43°C (LC agar at 27°C) for 72 h in media containing different inhibitory substances

Probiotic Culture		Media										
		MRS 37°C inc.	MRS-vancomycine 37°C inc.	MRS-vancomycine 43°C inc.	MRS-NNLP 37°C inc.	MRS-pH5.20 43°C inc.	MRS pH4.58 43°C inc.	MRS-NaCl (4%) 37°C inc.	MRS-LiCl (0.5%) 37°C inc.	LC agar 27°C inc.	MRS-bile (0.2%) 43°C inc.	MRS-bile 0.5% 43°C inc.
LB-	LB 100	9.3	<3.0	<3.0	<3.0	9.4	9.4	<3.0	<3.0	<3.0	<3.0	<3.0
ST-	TA 040	7.1	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0
ST-	DS 2362	8.1	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0
LC-	DS 930	9.5	9.4	<3.0	<3.0	<3.0	<3.0	9.1	9.1	9.1	<3.0	<3.1
LC-	LCO 1	9.4	9.2	<3.0	<3.0	<3.0	<3.0	9.1	9.2	9.1	<3.0	<3.2
LC-	LBC 81	9.3	9.2	<3.0	<3.0	<3.0	<3.0	9.3	9.1	9.3	<3.0	<3.3
LR-	LC 705	9.2	9.3	9.2	<3.0	9.1	9.1	9.0	9.0	9.5	5.5	<3.4
LR-	LBA	9.4	9.4	9.4	<3.0	9.4	9.3	6.4	<3.0	9.0	9.4	7.9
LA-	LA 5	7.1	<3.0	<3.0	<3.0	6.5	<3.0	<3.0	<3.0	<3.0	6.0	6.0
LA-	DS 910	8.3	<3.0	<3.0	<3.0	8.3	6.9	<3.0	<3.0	<3.0	8.1	<3.0
LA-	LAC 4	7.2	<3.0	<3.0	<3.0	6.0	<3.0	<3.0	<3.0	<3.0	6.3	5.0
LA-	74-2	7.2	<3.0	<3.0	<3.0	6.0	<3.0	<3.0	<3.0	<3.0	6.1	5.0
BB-	Bb 12	<3.0	<3.0	<3.0	7.4	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0	8.8
BB-	DS 920	<3.0	<3.0	<3.0	9.2	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0
BB-	420	<3.0	<3.0	<3.0	7.4	<3.0	<3.0	<3.0	<3.0	<3.0	8.2	<3.0
PS-	PS 1	10.5	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0
PS-	10360	10.8	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0

LB = *L. delbrueckii* ssp. *bulgaricus*, LC= *L.* casei, LR = *L. rhamnosus*, LA = *L. acidophilus*, BB = *Bifidobacterium*, PS = *Propionibacterium freudenreichii* subsp. *shermanii*.

Table 3.3 Counts (log_{10} cfu g^{-1}) and colony size (mm) of propionibacteria and other cultures in different bacteriological media after 7days

Cultures	MRS		NaLa agar		Arabinose agar		Raffinose agar		Xylose agar		LC agar		*NaLa agar, day7- NaLa agar day3
	count	colony Size mm	count	Colony Size mm	count	colony Size mm	count	colony Size mm	count	Colony Size mm	count	colony Size mm	count
LC- DS930	9.5	2.0	9.4	0.5-1.0	9.2	1.0	9.3	1.0	8.3	1.0	9.1	1.5-2.0	<3.0
LC- LC01	9.4	2.0	8.8	0.5-1.0	9.2	1.0	9.3	1.0	9.3	1.0	9.1	1.5-2.0	<3.0
LR- LC705	9.2	2.0	8.9	0.5-1.0	9.2	1.0	9.1	1.0	9.1	1.0	9.3	1.5-2.0	<3.0
LR- LBA	9.1	2.0	8.2	0.5-1.0	8.3	1.0	9.3	1.0	8.4	1.0	9.4	1.5-2.0	<3.0
LA- La5	7.1	0.1-0.5	6.8	0.1	7.7	0.1	<3.0	-	<3.0	-	<3.0	-	<3.0
LA- DS910	8.3	0.5-1.0	7.4	0.1	9.1	0.1	<3.0	-	<3.0	-	<3.0	-	<3.0
LA- 74-2	7.2	0.1-0.5	6.8	0.1	7.4	0.1	<3.0	-	<3.0	-	<3.0	-	<3.0
BB- Bb 12	<3.0	-	<3.0	-	<3.0		<3.0	-	<3.0	-	<3.0	-	<3.0
BB- DS920	<3.0	-	<3.0	-	<3.0	-	<3.0	-	<3.0	-	<3.0	-	<3.0
PS- PS1	10.5	0.1-0.5	9.8	1.5-2.0	9.6	1.0	9.7	0.5	9.6	0.5	<3.0	-	9.8
PS- 10360	10.8	0.1-0.5	9.9	1.0-2.5	8.7	1.0	9.4	0.5	8.4	0.5	<3.0	-	9.9

*-- Subtraction method

LB - *L. delbrueckii* ssp. *bulgaricus*, ST - *S. thermophilus*, LA- *L. acidophilus*, LC- *L.* casei,

LR- *L. rhamnosus*, BB- *Bifidobacterium*, PS - *Propionibacterium freudenreichii* subsp. *shermanii*.

Table 3.4 Viable counts ($\log_{10}$ cfu g^{-1}) of bacterial cultures in different sugar based media under aerobic incubation at 43°C for 72h.

Cultures	Sugar media				
	MRS	BA[1]-Sorbitol	BA- Manitol	BA-Maltose	BA-esculin
LB- LB 100	8.0	<3.0	<3.0	<3.0	<3.0
ST- DS 2362	<3.0	<3.0	<3.0	<3.0	<3.0
ST- TA 040	<3.0	<3.0	<3.0	<3.0	<3.0
LC- LCO 1	<3.0	<3.0	<3.0	<3.0	<3.0
LC- LBC 81	<3.0	<3.0	<3.0	<3.0	<3.0
LR- LC 705	9.3	9.3	9.2	9.3	9.1
LR- LBA	9.4	9.3	9.3	9.4	9.5
LA- DS 930	<3.0	<3.0	<3.0	<3.0	<3.0
LA-LA 5	6.5	<3.0	<3.0	<3.0	<3.0
LA- DS 910	7.6	<3.0	<3.0	7.1	7.3
LA- 74-2	8.9	<3.0	4.0	8.3	8.5
BB- Bb 12	<3.0	<3.0	<3.0	<3.0	<3.0
BB- DS 920	<3.0	<3.0	<3.0	<3.0	<3.0
BB- 420	<3.0	<3.0	<3.0	<3.0	<3.0
BB- BL	<3.0	<3.0	<3.0	<3.0	<3.0

LB = *L. delbrueckii* ssp. *bulgaricus*, ST = *S. thermophilus*, LA= *L. acidophilus*,

LC = *L.* casei, LR= *L. rhamnosus*, BB = *Bifidobacterium*

Table 3.5 Media recommended for selective enumeration of *Streptococcus thermophilus, Lactobacillus delbrueckii* spp. *bulgaricus, Lactobacillus acidophilus, Bifidobacterium, Lactobacillus casei, Lactobacillus rhamnosus,* and propionibacteria and viable counts of in a mixture of bacteria

Agar media	Bacteria	Incubation conditions	Colony morphology	Counts in a mixture of bacteria (cfu/mL)
S. thermophilus agar	*S. thermophilus*	Aerobic, 37^{0}C, 24h	0.1 – 0.5 mm, round yellowish	3.9 x 10^4
MRS[1] agar (pH 4.58)	*L. delbrueckii* spp. *bulgaricus*	Anaerobic, 45^{0}C, 72h	1.0 mm, white, cottony, rough, irregular	7.0 x 10^7
BA – sorbitol agar	*L. acidophilus*	Anaerobic, 37^{0}C, 72h	Rough, dull, small (0.1-0.5) brownish	10.0 x 10^7
MRS – NNLP[2] agar	Bifidobacteria	Anaerobic, 37^{0}C, 72h	1 mm, white shiny, smooth	7.0 x 10^7
MRS – vancomycine agar[3]	*L. casei*	Anaerobic, 37^{0}C, 72h	1.0 mm, white shiny, smooth	5.3 x 10^7
MRS – vancomycine agar	*L. rhamnosus*	Anaerobic, 43^{0}C, 72h	1.0-2.5 mm, white shiny, smooth	7.6 x 10^7
Sodium lactate agar	Propionibacteria	Anaerobic, 30^{0}C, 7-9d	1.0-2.5 mm, dull brown, lighter margin	4.9 x 10^7

[1]deMan, Rogosa, and Sharpe agar

[2]Nalidixic acid, neomycine sulfate, lithium chloride, and paromomycine sulfate

[3] If *L. rhamnosus* was not present; however, if *L. rhamnosus* was present, then subtraction methods could be used (i.e., subtracting *L. rhamnosus* counts on MRS-vancomycine agar under anaerobic incubation at 43°C for 72h from total counts of *L. casei* and *L. rhamnosus* obtained in MRS vancomycine agar under anaerobic incubation at 37^{0}C for 72h).

[4]Subtraction method could also be used to determine the counts of propionibacteria (i.e., counts of *L. casei* and *L. rhamnosus* (anaerobic incubation. 30^{0}C, 72h) could be subtracted from counts of *L. casei, L. rhamnosus,* and propionibacteria (anaerobic incubation, 30^{0}C, 7d)

Table 3. 6 Recovery of organisms from commercial products.

Products	Organisms claimed to be present	Organisms actually found						
		L. bulgaricus[1]	*S. thermophilus*[2]	*L. acidophilus*[3]	*L. casei*[4]	*L. rhamnosus*[5]	*Bifidobacterium lactis*[6]	*Propionibacterim*[7]
Product 1 (yoghurt)	Yoghurt culture	<3.00	8.69	<3.00	<3.00	<3.00	<3.00	<3.00
Product 2 (natural Yoghurt)	Yoghurt culture, *L. acidophilus, Bifidobacterium,* and *L. rhamnosus* GG	<3.00	9.17	5.23	<3.00	7.36	7.15	<3.00
Product 3 (flavoured yoghurt)	Yoghurt culture, *L. acidophilus, Bifidobacterium,* and *L. rhamnosus* GG	<3.00	9.01	6.53	<3.00	7.72	7.40	<3.00
Product 4 (natural yoghurt)	Yoghurt culture, *L. acidophilus, Bifidobacterium* and *L. casei*	7.68	8.83	7.83	5.53	<3.00	6.54	<3.00
Product 5 (skinny yoghurt)	Yoghurt culture, *L. acidophilus, Bifidobacterium* and *L. casei*	4.92	8.62	5.50	4.01	<3.00	6.36	<3.00
Product 6 (Swiss cheese)	Cheese culture	<3.00	<3.00	<3.00	<3.00	<3.00	<3.00	3.59

[1]Enumerated using MRS agar (pH 5.58)

[2]Enumerated using *S. thermophilus* agar

[3]Enumerated using BA-sorbitol agar

[4]Enumerated using subtraction method (counts in MRS- vancomycine agar at 37°C – counts in MRS- vancomycine agar at 43°C)

[5]Enumerated using MRS-vancomycine agar at 43°C

[6]Enumerated using MRS_NNLP agar

[7]Enumerated using subtraction method (counts in NaLa agar at day 7 – counts in NaLa agar at day 3)

CHAPTER 4

SURVIVAL OF *LACTOBACILLUS ACIDOPHILUS, LACTOBACILLUS PARACASEI* SUBSP. *PARACASEI, LACTOBACILLUS RHAMNOSUS, BIFIDOBACTERIUM ANIMALIS* AND *PROPIONIBACTERIUM* IN CHEESE-BASED DIPS AND THE SUITABILITY OF DIPS AS EFFECTIVE CARRIERS OF PROBIOTIC BACTERIA

4.1 Introduction

A number of food products including probiotic yoghurt, yoghurt like cereal products, drinking yoghurt, power drinks, kefir, ice cream (Haynes and Playne, 2002), frozen fermented dairy deserts (Ravula and Shah, 1998a), freeze-dried yoghurt (Rybka and Kailasapathy, 1995), probiotic Cheddar cheese (Stanton *et al.*, 2001), spray dried milk powder (Stanton *et al.*, 2001) fruit and berry juices and coleslaw (Rodgers and Odongo, 2002) have been employed as delivery vehicles for probiotics. Resistant starch has been proven to improve the survival of probiotics in yoghurt (Brown *et al.,* 1998) and in low fat ice cream (Haynes and Playne, 2000). But due to the presence of hydrogen peroxide, high acid levels, inhibitory substances produced by yoghurt bacteria (Shah and Lankaputhra, 1997; Dave and Shah, 1997), high oxygen content (Lankaputhra and Shah 1997) in the product, injury due to freezing (Lankaputhra, Shah, and Britz, 1996) and freeze drying (Rybka and Kailasapathy, 1995), many of the above mentioned products have failed to successfully deliver the required level of viable cells of probiotics.

Cheese-based dips could be a delivery vehicle for probiotic bacteria owing to its stable pH, buffering capacity of ingredients used and the presence of prebiotics. At an average consumption of about 50 - 100 g per serving of dips (Black Swan and Poseidon Dips Pty. Ltd., Victoria, Australia), they can be an effective delivery media for probiotic bacteria, independently or as complementary to other probiotic products.

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However, little is known about the survival of probiotic bacteria in dips during their shelf life. Ingredients used to improve the texture, safety (pH) and organoleptic qualities of dips such as organic acids (acetic acid, lactic acid and citric acid) and, oil and gums may affect the survival of probiotic bacteria in dips. The potential of improving the microenvironment of the dip in order to improve viability of probiotic bacteria is also needs to be investigated.

The aim of this study was to establish the suitability of cheese-based dips as a delivery vehicle for probiotic bacteria such as *L. acidophilus, L. paracasei* subsp. *paracasei, L. rhamnosus, B. animalis*, and *P. freudenreichii* subsp. *shermanii.* The specific objectives were firstly to identify the best combination of probiotic bacteria that produces maximum cell numbers in dips and then to ascertain the effects of standard dip ingredients such as organic acids, oils and gums and food additives such as L-cysteine and $NaHCO_3$ on the survival of probiotics in dips.

4.2. Material and methods

4.2.1 Experimental design and treatments

The study consisted of a sequence of four experiments. The first experiment was designed to select the best combination of five strains of probiotic bacteria in terms of survival in French Onion dip. The preperation of the dips is described in section 4.2.2. The probiotic bacteria used were:

1. *Lactobacillus acidophilus* **(A)**
2. *Bifidobacterium animalis* **(B)**
3. *Lactobacillus paracasei* subsp. *paracasei* **(C)**
4. *Lactobacillus rhamnosus* **(R)**
5. *Propionibacterium freudenreichii* subsp. *shermanii* **(P)**

A and B were selected because their probiotic properties are well established. C and R were selected based on the recent discovery of their probiotic and therapeutic properties. Since, C and R are suggested to have similar qualities, they were used mutually exclusively in combinations. P was selected for its capacity to produce vitamin B, which is suggested to improve the quality of the dip and the growth of other probiotic bacteria. The treatments included the five bacteria (as controls) and eight strategic bacterial

combinations (ABCP, ABRP, ABC, ABR, BCP, BRP, BC and BR). The combinations of bacteria used in the experiment are shown in Table 4.1.

The second experiment was designed to determine the effect of type of acid and pH on the survival of probiotic bacteria. The experimental treatments comprised of a factorial combination of three acid types (acetic, lactic and citric acids) and three pH levels (4.45, 4.30 and 4.20). A control treatment with pH 4.45 (legal product requirement) and with a mixture of all three acids (in equal proportions) was included for comparison. Each treatment was replicated twice.

The third experiment determined the effect of addition of canola oil with or without gum (a combination of CMC and xanthan gums) to dips on the survival of probiotic bacteria. The treatments were; oil, oil + gum and control (neither). Each treatment was replicated twice.

The fourth experiment determined the effect of addition of L-cysteine hydrochloride or sodium bicarbonate on the survival of two selected combinations of probiotic bacteria (factorial combinations of two bacterial types and two chemicals).

4.2.2 Preparation of the dip

The experimental dip was made according to the formula and methodology adopted by Poseidon and Black Swan Dips, Victoria, Australia for French onion dip. The composition of the dip included (% by weight): cream cheese (62), onion (11), water (20) and minor ingredients (canola oil, lemon juice, vinegar, lactic acid), thickeners and herbs and spices. Immediately after the blending of ingredients, the dip was stored at 4°C before being used in the experiment. For experiments 2, 3 and 4, a base dip was made without the test material (control). Bacterial cultures and organic acids, oil/ gums, L-cysteine hydrochloride or sodium bicarbonate were then added to the base dip.

4.2.3 Probiotic bacterial cultures

Cultures of *L. acidophilus* (LAC1) and *L. paracasei* subsp. *paracasei* (LCS1) were obtained from DSM (DSM Food Specialties, Australia Pty. Ltd., Werribee, Australia). *B. animalis* (Bb12) and *P. freudenreichii* subsp. *shermanii* (PS1) were received from Chr. Hansen (Chr. Hansen Pty. Ltd. Bayswater, Australia). *L. rhamnosus* (LC 705) was obtained from Bronson and Jacob (Bronson and Jacob, Dingley, Australia). Before use,

all organisms were tested for purity using Gram stain and sugar utilization patterns. The starter cultures were in freeze-dried (DVS) form or frozen (DVS) form. The storage and maintenance of the cultures was carried out as per the recommendation of the manufacturers.

4.2.4. Preparation of media

4.2.4.1. Bacteriological peptone and water diluent

This medium was prepared as outlined by Tharmaraj and Shah (2003) and discribed in sectrion 3.2.2.

4.2.4.2. MRS-NNLP agar

This medium was prepared as outlined by Tharmaraj and Shah (2003) and discribed in sectrion 3.2.2.

4.2.4.3. MRS-vancomycin agar, BA-*sorbitol agar*, *sodium lactate (Na La) agar*

These media were prepared as outlined by Tharmaraj and Shah (2003) and discribed in sectrion 3.2.2.

4.2.5. Enumeration of bacteria

Ten grams of dip was mixed with 90 ml of 0.15% sterile bacteriological peptone followed by mixing homogeneously using a stomacher and 10-fold serial dilution (10^3 to 10^7) were prepared. The enumeration was carried out using the pour plate technique. Duplicate plates were incubated anaerobically at 37°C for 72 h in a gas mixture of 10% CO_2, 5% H_2 and 85% N_2 in anaerobic jars using gas generating kits (Anaerobic System BR 38; Oxoid Ltd., Hampshire, England). Plates containing 25 to 250 colonies were enumerated and recorded as colony forming units (CFU) $gram^{-1}$of the product or culture. The enumeration of *L. acidophilus*, *L. paracasei* subsp. *paracasei*, *L. rhamnosus* and *P. freudenreichii* subsp. *shermanii* was carried out as described by Tharmaraj and Shah (2003). *L. acidophilus*, *L. paracasei* subsp. *paracasei* and *L. rhamnosus* were enumerated on MRS-sorbitol agar. Colonies that were irregular in shape with a diameter of 0.1-0.5 mm were counted as *L. acidophilus*. White smooth shiny disc-like colonies with a

diameter of 1-2 mm were counted as of *L. paracasei* subsp. *paracasei* or of *L. rhamnosus*. *L. paracasei* subsp. *paracasei* and *L. rhamnosus* were also enumerated on MRS-vancomycin agar. Since *L. paracasei* subsp. *paracasei* and *L. rhamnosus* have not been added together in any of the combinations, the subtraction method described by Tharmaraj and Shah (2003) have not been used. *B. animalis* was counted on MRS-NNLP agar as described by Laroia and Martin (1991) and *P. freudenreichii* subsp. *shermanii* was counted on NaLa (sodium lactate) agar. Brownish smooth shiny lenticulate colonies with a diameter of 1-3 mm were counted as *P. freudenreichii* subsp. *shermanii*.

4.2.6. Experiment 1- Survival of probiotic bacteria in dip

The different bacterial consortium (8 combinations + 5 controls), in two replicates, was inoculated to 2.5 kg lots of French onion dips at a level of log 7 cfu g^{-1} (notionally) and mixed well aseptically in a laboratory mixer. The dips (26 types) were then packed in 150 g portions in non-transparent plastic containers, sealed airtight and stored at 4°C for a period of 10 weeks. Duplicate samples were collected from each dip at 2 weeks interval. The duplicate samples from each replicate were bulked, mixed homogeneously and a sub-sample was aseptically taken for microbiological count. The pHof the dip was 4.4 ± 0.02.

4.2.7. Experiment 2- Effect of pH and type of organic acids on the survival of probiotic bacteria

From the results of the first experiment, the bacterial combination ABCP was selected for this experiment. Initially, a base dip was prepared as outlined before omitting acids. The four bacterial cultures (*L. acidophilus* (A), *B. animalis* (B), *L. paracasei* subsp. *paracasei* (C) and *P. freudenreichii* subsp. *shermanii* (P)) were inoculated at a rate of 10^7 CFU $gram^{-1}$ in each of the ten batches of dips (three acid types x three pH levels + control). Citric acid (10.0, 15.0 and 20.0 ml of 10% solution of citric acid kg^{-1}) acetic acid (2.0, 3.0 and 4.0 ml of acetic acid kg^{-1}) or lactic acid (1.25, 2.0 and 2.5 ml of lactic acid kg^{-1}) were added separately to bring the pH to three different levels (4.45, 4.30 and 4.20). The probiotic dips were mixed homogeneously, and packed and sealed airtight in 150g portions. The sealed containers were stored at 4°C for a period of 10 weeks. Starting from

day 2, duplicate samples were collected at 2-week intervals for 10 weeks, from each batch of dip for analysis. Duplicate samples from each replicate were mixed homogeneously and a sub-sample was aseptically taken for microbiological analysis. The rest of the samples were used to measure pH.

4.2.8. Experiment 3- Effect of oil and gums on the survival of probiotic bacteria

Initially, 5 kg of French onion dip was made without oil and gums. Canola oil or gum was added to the base dip and four bacterial cultures consisting of ABCP (*L. acidophilus* (A), *B. animalis* (B), *L. paracasei* subsp. *paracasei* (C) and *P. freudenreichii* subsp. *shermanii* (P)) were inoculated at a rate of 10^7 cfu g^{-1}, mixed homogeneously, and packed and sealed airtight in 150 g portions. The sealed containers were stored at 4°C for a period of 10 weeks. Starting from day 2, duplicate samples were collected at 2-weeks intervals for 10 weeks, from each treatment for analysis. The duplicate samples of each treatment were mixed homogeneously and a sub-sample was aseptically taken for microbiological count.

4.2.9. Experiment 4- Effect of L-cysteine hydrochloride and sodium bicarbonate on the survival of probiotic bacteria

Since *P. freudenreichii* subsp. *shermanii* was found to survive well in dips in all of the earlier experiments, this organism was not selected for this experiment. The combinations ABC (*L. acidophilus* (A), *B. animalis* (B), *L. paracasei* subsp. *paracasei* (C)) and ABR (*L. acidophilus* (A), *B. animalis* (B), *L. rhamnosus* (R)) were used in this study. L-cysteine was added to reduce the oxidation-reduction potential of the dip. Sodium bicarbonate was added to neutralize the acid effect and to produce HCO_3^- and CO_2. The additives, were mixed with the dip at the rate of 0.05% by weight. Each of the two types of probiotic dips (ABC and ABR) was prepared by mixing the respective bacterial cultures to French onion dip and packed in 150 g portions in plastic containers. The sealed containers were stored at 4°C for 10 weeks. Enumeration of bacteria was performed at day 2 (week 0), week 2, week 4, week 6, week 8, and week 10.

4.2.10. Statistical analysis

The results were analysed by General Analysis of Variance model using the GENSTAT program (Genstat committee, 1995). In experiment 1, each bacterial type was analysed and presented separately for differences between individual bacterial type (control) and its combinations. Means were compared using the least significant difference (LSD). In experiment 2, only the data belonging to the three types of acids with pH 4.45 and control was subjected to statistical analysis.

4.3. Results and discussions

4.3.1. Effects of bacterial combinations on the survival of probiotic bacteria in dips

The changes in bacterial population over 10 weeks of refrigerated storage of *L. acidophilus, B. animalis, L. paracasei* subsp. *paracasei, L. rhamnosus and P. freudenreichi* subsp. *shermanii* are given in Tables 4.2-4.6. Since the number of combinations of each of the five bacteria was different, the data of each bacterium was analysed and presented separately. When the average counts of individual bacterial types (the 5 control treatments) were compared, the order of bacterial type in terms of survival over 10 weeks period ($P<0.001$) was *L. paracasei* subsp. *paracasei* > *L. rhamnosus* > *P. freudenreichi* subsp. *shermanii* > *L. acidophilus* = *B. animalis* (Tables 4.2– 4.6). The survival of *L. acidophilus* (A) in control (log 6.3) was less than that of combinations, but was not significantly affected by the bacterial combination (log 6.5-6.7, Table 4.2). However, irrespective of the bacterial combination, the population of *L. acidophilus* declined over storage at an average rate of 0.01 log unit per day, and lost about 0.9 log of population over 10 weeks. Initially, the population dropped by 11.7% in 2 days and then at a slow rate of around 0.9% per 2 weeks. The viable population of 6.35 log g^{-1} after 10 weeks resulted from an initial inoculation rate of 7.39 log. Since dips are consumed at relatively smaller quantities (50- 100 g/serving), a higher inoculation rate of around 9 log g^{-1}) of L. *acidophilus* may be needed to maintain and deliver the beneficial population levels for 10 weeks. Although, the survival of *L. acidophilus* did not vary significantly between bacterial combinations, the combinations without *P. freudenreichi* subsp. *shermani* (ABC and ABR) contained greater populations of *L. acidophilus* compared to

those with this organism (ABCP and ABRP). The results also indicate that *L. acidophilus* survives better in the presence of *L. paracasei* subsp. *paracasei* than *L. rhamnosus*, and that ABC or ABCP provided the best bacterial combination for the survival of *L. acidophilus*.

The survival of *B. animalis* (B) was significantly ($P<0.01$) affected by the bacterial combination and during storage ($P<0.01$, Table 4.3). The population of *B. animalis* dropped by about 1 log in the first week, remained static until week 4 and thereafter declined slowly by about 0.8-1.0 log between week 4 and week 10. However, there was a significant ($P<0.01$) interaction between bacterial type and storage days in the changes in *B. animalis* population. In all four combinations in which *L. acidophilus* was present, the initial drop in the population of *B. animalis* was relatively smaller than that in combinations without *L. acidophilus*. In the absence of *L. acidophilus*, the population of *B. animalis* dropped well below the critical level of log 6 cfu g^{-1} (Kurman and Rasic, 1991) within the first week (Table 4.3). In combinations with *L. acidophilus*, the population of *B. animalis* remained above the lower critical level for up to 6 weeks. Out of the four bacterial combinations that contained *L. acidophilus*, the survival of *B. animalis* was highest in ABCP combination during the 10-week storage. Out of all combinations, the count of *B. animalis* was the least in BR. All combinations of *B. animalis* with *L. paracasei* subsp. *paracasei* contained almost similar *B. animalis* population at the end of 10 weeks (Table 4.3). A reduction in log population of 1.55 to 1.70 to that of the initial population was found in these combinations, whereas the combinations with *L. rhamnosus* showed reduction of up to 2 log cycles. The presence of *P. freudenreichii* subsp. *shermanii* did not appear to affect the final population at 10 weeks. These findings indicate that *L. paracasei* subsp. *paracasei* and *L. rhamnosus* might inhibit *B. animalis*, while the effect of *L. rhamnosus* might be more than that of *L. paracasei* subsp. *paracasei*. These findings were further confirmed by the experiments carried out on the inhibitory effects within probiotics (data not shown). However, in BRP and BC combinations, after a drastic initial drop, the population of *B. animalis* remained static throughout the 10 weeks storage period. This suggests that at a higher initial inoculation level (log 9-10), *B. animalis* may survive above required levels for a longer time.

Survival of *L. paracasei* subsp. *paracasei* (C) was significantly affected by the bacterial combination ($P<0.05$) and days on shelf ($P<0.01$, Table 4.4). Though the population of *L paracasei* subsp. *paracasei* declined at day 2, thereafter, it increased during 10 weeks storage. The population growth pattern of *L. paracasei* subsp. *paracasei* varied significantly ($P<0.01$) with time between bacterial combinations (Table 4.4). In ABC and BCP combinations, *L. paracasei* subsp. *paracasei* started to grow above the initial level by week 2 but thereafter remained relatively static until week 10. In ABCP, the population of *L. paracasei* subsp. *paracasei* remained relatively static from week 0 to week 4 and thereafter increased by about one log cycle. This increase in the population of *L. paracasei* subsp. *paracasei* was greater in the ABC combination than ABCP one. The increase in *L. paracasei* subsp. *paracasei* population remained after week 6 in ABCP combination. In contrast, the population of *L. paracasei* subsp. *paracasei* declined below the initial level in BCP combination at week 10. The difference in the survival of *L. paracasei* subsp. *paracasei* in ABCP and BCP and ABC and BC combinations (Table 4.4) is suggestive of a beneficial effect of *L. acidophilus* on the growth and survival of *L. paracasei* subsp. *paracasei*. However, at the end of week 10, ABC and BC showed the highest population level of *L. paracasei* subsp. *paracasei*, nullifying the effect of *L. acidophilus*. When inoculated at a rate of 7.40 log, all the combinations contained well above the required population of log 6 cfu g^{-1} of *L. paracasei* subsp. *paracasei* throughout the storage.

Table 4.5 shows the survival of *L. rhamnosus* (R) population in four different bacterial combinations. The bacterial combination or storage did not significantly affect the survival of *L. rhamnosus*. However, *L. rhamnosus* grew well in the ABRP combination compared to the other combinations. In ABRP combination, the population of *L. rhamnosus* grew throughout the 10 weeks and reached a population that was about 0.2 log unit higher than the initial inoculation level.

The survival of *P. freudenreichii* subsp. *shermanii* (P) was not affected by the bacterial combination (Table 4.6). From week 0 to week 2 - 4, the population of this bacterium declined below 4 log units. Thereafter, *P. freudenreichii* subsp. *shermanii* started to grow steadily till the end of 10 weeks storage. This may indicate that the *P. freudenreichii* subsp. *shermanii* is able to grow at refrigeration temperature and might

either have grown some resistance to conditions prevailed in the dip that suppressed or inhibited initially or the resistant bacteria in the culture could have started to grow. The *P. freudenreichii* subsp. *shermanii* started to grow relatively earlier (week 4) in the presence of *L. acidophilus* in the combination (ABCP and ABRP) than in combinations without *L. acidophilus* (BCP and BRP). Further investigation is needed to establish the reason for the initial drastic drop in their populations.

Table 4.7 shows the rank of survival of each type of bacteria in different bacterial combinations. *L. acidophilus* (A) survived the best in the combination ABC followed by ABCP. *B. animalis* (B) survived the best in combination ABCP followed by ABC. Bacterial combinations did not affect *P. freudenreichii* subsp. *shermanii* (P). The combination ABC performed the best followed by ABCP, ABRP and ABR when *L. paracasei* subsp. *paracasei* (C) and *L. rhamnosus* (R) considered together. From the above results, the combination ABC could be selected as the best combination where the probiotics (*L. acidophilus* (A), *B. animalis* (B) and *L. paracasei* subsp. *paracasei* (C) performed the best. The combination ABCP (*L. acidophilus* (A), *B. animalis* (B) and *L. paracasei* subsp. *paracasei* (C) and *P. freudenreichii* subsp. *shermanii* (P) could be selected as the second best. These results suggest that *L. acidophilus* is contributing positively to the survival of *B. animalis*, *L. paracasei* subsp. *paracasei* and *L. rhamnosus*. *B. animalis* appeared to be antagonistic to *L. paracasei* subsp. *paracasei* and *L. rhamnosus*. *B. animalis* and *P. freudenreichii* subsp. *shermanii* appeared to have a negative effect on *L. paracasei* subsp. *paracasei* and *L. rhamnosus*. This negative effect appears to be additive as in the presence of both *B. animalis* and *P. freudenreichii* subsp. *shermanii*, the survival rank (Table 4.7) of the combinations of BCP (*L. paracasei* subsp. *paracasei*, *B. animalis* and *P. freudenreichii* subsp. *shermanii*) and BRP (*L. rhamnosus*, *B. animalis* and *P. freudenreichii* subsp. *shermanii*) were pushed to the last. The positive effect of *L. acidophilus* on the other bacteria appears to be strong to overpower and nullify the antagonistic effects of *B. animalis* on *L. rhamnosus* and *L. paracasei* subsp. *paracasei*. The antagonistic effect of *B. animalis* and *L. rhamnosus* and/ or *L. paracasei* subsp. *paracasei* appears to be mutual (Table 4.3). The population of *B. animalis* was the least in combinations of BC (*B. animalis* and *L. paracasei* subsp. *paracasei*) and BR (*B. animalis* and *L. rhamnosus*) indicating that *L. paracasei* subsp. *paracasei* and *L.*

rhamnosus have affected the population of *B. animalis* negatively. The antagonistic effects of these probiotic bacteria need further investigation. *P. freudenreichii* subsp. *shermanii* did not appear to interfere with any of the other bacteria.

4.3.2. Effect of pH and type of organic acid on the survival of probiotic bacteria

Lactic acid, acetic acid and citric acid are naturally occurring and most commonly used organic acids to enhance organoleptic qualities as well as safety of many food products. Lactic acid bacteria are found to be more tolerant to acidity and organic acids than most of the pathogens and spoilage microorganisms. The effect of organic acids at different pH levels of 4.45, 4.30, 4.20 on the population of probiotic bacteria is shown in Table 4.8. In general, the bacterial population dropped at week 2 and thereafter increased until week 10. On average, *L. paracasei* subsp. *paracasei* (6.6 log) and *P. freudenreichii* subsp. *shermanii* (6.7 log) survived better than *L. acidophilus* (5.9 log) and *B. animalis* (5.8 log) over the 10 weeks of storage period.

L. acidophilus and *B. animalis* were not significantly affected at pH levels of 4.45, 4.30 and 4.20 in any of the tested organic acids after the initial reduction of 1 log at the end of two weeks. The initial reduction might be due to the initial higher temperature and acidity of the product or due to the antagonism among probiotic bacteria, while the metabolic activity was higher before the product reached the storage temperature of 4°C (Table 4.8). It has been reported that *L. acidophilus* and *B. animalis* are affected by pH 5.0 (Shah, Lankaputhra, Britz and Kyle, 1995; Lankaputhra and Shah 1997). The information on antagonistic effects among *L. acidophilus*, *B. animalis*, *L. paracasei* subsp. *paracasei* and *P. freudenreichii* subsp. *shermanii* is limited. After 2 weeks, the level of reduction with acetic acid was higher than the other two acids for *L. acidophilus*. *B. animalis* performed better in acetic acid than in the other acids. This might be due to the reduced antagonistic effect of the other bacteria that were inhibited by acetic acid or *B. animalis* might be more resistant to acetic acid than other bacteria since acetic acid is one of the metabolites of this bacterium.

L. paracasei subsp. *paracasei* was not adversely affected by any acids or at any pH levels, and the organisms showed an increase in the population of 0.5 to 1 log in lactic and citric acid at the pH of 4.45- 4.20. In acetic acid, this bacterium showed varied levels

of reduction in their population. Acetic acid might have inhibited *L. paracasei* subsp. *paracasei* slightly (Table 4.8). This inhibitory effect of acetic acid on *L. paracasei* subsp. *paracasei* might be the reason for the effect of *B. animalis* observed in the earlier experiment (Table 4.4). But *L. paracasei* subsp. *paracasei* performed slightly better in pH 4.20 than either in pH of 4.45. This might be due to the antagonistic effect of co-bacteria that tolerated acetic acid slightly better than *L. paracasei* subsp. *paracasei*, possibly *B. animalis*. At pH 4.30, the antagonistic co-bacteria might have lost its tolerance to acetic acid and control over *L. paracasei* subsp. *paracasei,* allowing it to show better survival. At pH 4.20, *L. paracasei* subsp. *paracasei* appeared to have been affected by acetic acid. The overall excellent survival of *L. paracasei* subsp. *paracasei* might indicate that *L. paracasei* subsp. *paracasei* is resistant to higher acid levels or the organism did not suffer bacterial antagonism or dominated the niche by suppressing other probiotic bacteria present. This speculation needs to be verified.

P. freudenreichii subsp. *shermanii* population observed to have reduced by 2 log cycle by the end of week 2 but after week 2 this organism showed continuous growth till the end of the storage. In all treatments (Table 4.8), the initial suppression of *P. freudenreichii* subsp. *shermanii* might be due to bacterial antagonism during the high metabolic activity of co-bacteria during the early incubation period. The population growth after week 2 might indicate the ability of *P. freudenreichii* subsp. *shermanii* to grow and proliferate at lower storage temperatures and at lower pH levels, where the metabolic activities of other bacteria are at their minimal.

Table 4.8 shows that the treatment control, which had all three organic acids and a pH of 4.45 supported all probiotic bacteria better than single acid treatments. Antimicrobial property of acids depends on temperature, pK, concentration and pH along with its lipophilic property and solubility. Acetic acid is the most lipophilic out of the three acids. pK is the pH at which concentrations of the un-dissociated molecules and the dissociated molecules are equal. The pK values of acetic, lactic and acids citric are 4.80, 3.86 and 3.06, respectively. The acetic acid with higher pK value might have had higher proportion of un-dissociated molecules that are more lipophilic and antimicrobial. This might be another reason for the suppression of probiotics shown in the combinations with acetic acid. When the concentration of acetic acid was reduced in the control dip by the

inclusion of other acids (acetic acid: lactic acid: citric acid = 1:1:1), though the pH was similar, the suppressive effect was reduced. The drastic reduction observed during the first 2 weeks might be due to the temperature effect with acid effect, where the temperature dropped from room temperature to 4°C. At room temperature, the suppression effect of acids might have been more than at 4°C.

The pH of the experimental dips over a period of 10 weeks is shown in Figure 4.1. In all the treatments, the pH was observed to reduce during the first few weeks and then stayed constant or started to increase slowly. The reduction was the highest (0.15 pH units) in treatments with the highest pH of 4.45, all other treatments showed a reduction of 0.08- 0.10 pH unit. The control showed a reduction of 0.11 unit at the beginning and after that the pH stayed constant. The reduction in the pH during the first few weeks of the storage might be due to the metabolic activity and acid production by the probiotic bacteria. The higher reduction at pH 4.45 might indicate the highest metabolic activity of the organisms at this pH. The slight increase in pH might be due to the metabolism of the acids during the growth and proliferation of *P. freudenreichii* subsp. *shermanii*. The pH pattern again might be an indication of microbial metabolic activity during the first two days, where acids might have been produced to reduce the pH. The reduction of all probiotic bacterial population occurred during this period of high metabolic activity. The increase in pH observed following the drop might be due to the metabolism of acids by the growth of *P. freudenreichii* subsp. *shermanii*.

4.3.3. The effect of oil and gum on the survival of probiotic bacteria

As observed in previous experiments, in all three treatments (oil and gum, oil only and control with no oil and no gum) populations of *L. acidophilus* and *B. animalis* dropped rapidly during first two days and continued to drop slowly to result around 0.8 log unit for *L. acidophilus* and 1.3 log units for *B. animalis*. Other than this, no statistical significance between treatments could be observed. The population of *L. paracasei* subsp. *paracasei* was maintained at the inoculated rate throughout the shelf life. Although *P. freudenreichii* subsp. *shermanii* showed better growth in the presence of oil and gum, this effect was not statistically significant.

4.3.4. Effect of L-cysteine·hydrochloride and $NaHCO_3$ on the survival of probiotic bacteria

On average, the bacterial population (averaged over 10 weeks) was greater in the ABC (*L. acidophilus* (A), *B. animalis* (B) and *L. paracasei* subsp. *paracasei)* combination (7.2 log) than in the ABR (*L. acidophilus* (A), *B. animalis* (B) *L. rhamnosus* (R)) combination (6.8 log), though the inoculation rate was the same. In both the combinations, the population of *B. animalis* was greater than *L. paracasei* subsp. *paracasei*, *L. rhamnosus* and *L. acidophilus*. This might be due to the higher inoculation level (8 log cfu g^{-1}) of *B. animalis* as speculated in the earlier experiment. The populations of all the bacteria, except R, were greater in dips with $NaHCO_3$ than L-cysteine and control. The higher population in dips with $NaHCO_3$ may be due to reduced acid effect as a result of buffering. Though L-cysteine was expected to increase the population of probiotic bacteria by reducing redox potential, the effect was observed to be the opposite. Dave and Shah (1997) observed the same effect with bifidobacteria. The changes in bacterial population over time in both bacterial combinations and treatments are shown in Figure 4.2. The population of *B. animalis* remained at similar levels in the control and with additives until week 2. After week 2, the population of *B. animalis* started to drop at a faster rate in dip with L-cysteine and at a relatively slower rate in dips with $NaHCO_3$. In both the bacterial combinations, the population of *L. acidophilus* dropped to 6 logs or below by week 2 and remained almost at that level till week 10. This might be due to the inhibition by *L. paracasei* subsp. *paracasei*, *L. rhamnosus* or *B. animalis*. However, in ABR combination the drop in *L. acidophilus* population was very drastic (<6 log) suggesting a greater inhibitory effect of *L. rhamnosus* on *L. acidophilus* than *L. paracasei* subsp. *paracasei*. The population of *L. paracasei* subsp. *paracasei* and *L. rhamnosus* was unaffected by additives or days in shelf.

4.4. Conclusion

This study has shown that French onion dip can be used as an effective carrier for probiotic bacteria when inoculated at 9 log g^{-1} or more. Bacterial combinations affected *L. acidophilus,* and *B. animalis*. When inoculated at 9 log g^{-1} or more, *L. acidophilus* and *B. animalis* population can be maintained above required level for health benefit over the

storage period of 10 weeks. *L. paracasei* subsp. *paracasei*, *L. rhamnosus* and *P. freudenreichii* subsp. *shermanii* were not adversely affected by any of the bacteria in any combinations and can be inoculated at a rate of 7 log to maintain a population above 6 logs over the storage period of 10 weeks. The antagonistic effect with *L. paracasei* subsp. *paracasei* and *L. rhamnosus* needs further investigation. Selecting probiotic combination that show little or no antagonistic effect towards each other and the level of inoculation are the critical factors to maintain high population levels of probiotic bacteria in the dip. The combinations with *L. acidophilus, B. animalis, L. paracasei* subsp. *paracasei* and *P. freudenreichii* subsp. *shermanii* and *L. acidophilus, B. animalis, L. paracasei* subsp. *paracasei* and *P. freudenreichii* subsp. *shermanii* and *L. rhamnosus* can be used as probiotic combinations. However, the combination with *L. paracasei* subsp. *paracasei* was better than the combination with *L. rhamnosus* as a probiotic consortium.

Table 4.1. The treatment combinations of probiotic bacteria used to study the survival of individual bacteria

Bacterial combination	Probiotic bacteria				
	Lactobacillus acidophilus (A)	*Bifidobacterium lactis (B)*	*Lactobacillus para casei(C)*	*Lactobacillus rhamnosus(R)*	*Propioni bacterium freudenreichii(P)*
ABCP	✓	✓	✓		✓
ABRP	✓	✓		✓	✓
ABC	✓	✓	✓		
ABR	✓	✓		✓	
BCP		✓	✓		✓
BRP		✓		✓	✓
BC		✓	✓		
BR		✓		✓	

Table 4.2. Changes in $\log_{10}$ population (cfu g^{-1}) of *Lactobacillus acidophilus* in dips with different culture combinations over 10 weeks of storage

Time in shelf (weeks)	Bacterial combination				
	ABCP	ABRP	ABC	ABR	A (Control)
0 (initial)	7.4	7.4	7.4	7.4	7.4
1	6.5	6.4	6.6	6.6	6.5
2	6.5	6.5	6.7	6.8	6.4
4	6.4	6.3	6.6	6.5	6.1
6	6.3	6.4	6.5	6.5	6.0
8	6.3	6.5	6.4	6.4	6.0
10	6.4	6.4	6.4	6.2	5.8
Average	*6.5*^{a}	*6.6*^{a}	*6.7*^{a}	*6.6*^{a}	*6.3*^{b}
$LSD_{0.05}$	*0.14* **				

$LSD_{0.05}$ = Least significant difference at P<0.05.

** Means significantly different at P<0.001. Means with different superscripts within row differs significantly at P<0.05.

ABCP- *L. acidophilus, B. animalis, L. paracasei* subsp. *paracasei, P. freudenreichii*

ABRP- *L. acidophilus, B. animalis, Lactobacillus rhamnosus, P. freudenreichii*

ABC- L. acidophilus, B. animalis, L. paracasei subsp. *paracasei*

ABR- L. acidophilus, B. animalis, L. rhamnosus

A- *L. acidophilus*

Table 4.3. Changes in $\log_{10}$ population (cfu g^{-1}) of *Bifidobacterium animalis* in dips with different bacterial combinations over 10 weeks of storage

Storage time (weeks)	Bacterial combination								
	ABCP	ABRP	ABC	ABR	BCP	BRP	BC	BR	B (Control)
0 (initial)	7.4	7.4	7.4	7.4	7.4	7.4	7.4	7.4	7.4
1	6.4	6.5	6.6	6.6	5.7	5.7	5.9	5.7	6.9
2	6.5	6.5	6.6	6.7	5.7	5.8	5.8	5.7	6.6
4	6.5	6.4	6.5	6.5	5.7	5.8	5.8	5.4	6.5
6	5.9	6.0	5.9	6.0	5.9	6.0	5.9	5.8	6.1
8	6.0	5.8	5.8	5.8	5.9	6.0	5.8	5.4	5.5
10	5.7	5.5	5.6	5.5	5.6	6.0	5.8	5.4	5.0
Average	6.4^{a}	6.3^{ab}	6.4^{a}	6.4^{a}	6.0^{d}	6.1^{c}	6.0^{c}	5.9^{e}	6.3^{b}
$LSD_{0.05}$	0.07**								

$LSD_{0.05}$ = Least significant difference at P<0.05.

** Means significantly different at P<0.001. Means with different superscripts within row differs significantly at P<0.05.

ABCP- *L. acidophilus, B. animalis, L. paracasei* subsp. *paracasei, P. freudenreichii*

ABRP- *L. acidophilus, B. animalis, L. rhamnosus, P. freudenreichii*

ABC- *L. acidophilus, B. animalis, L. paracasei* subsp. *paracasei*

ABR- *L. acidophilus, B. animalis, L. rhamnosus*

BCP- *B. animalis, L. paracasei* subsp. *paracasei, P. freudenreichii*

BRP- *B. animalis, L. rhamnosus, P. freudenreichii*

BC- *B. animalis, L. paracasei* subsp. *paracasei*

BR- *B. animalis, L. rhamnosus*

B- *B. animalis*

Table 4.4 Changes in $\log_{10}$ population (cfu g^{-1}) of *Lactobacillus paracasei* subsp. *paracasei* in dips with different bacterial combinations over 10 weeks of storage

Storage time (weeks)	Bacterial combination				
	ABCP	ABC	BCP	BC	C (Control)
0 (initial)	7.4	7.4	7.4	7.4	7.4
1	7.1	6.8	6.8	6.9	7.1
2	7.3	7.7	7.8	6.8	7.4
4	7.2	7.7	7.6	7.7	7.5
6	8.1	7.9	7.7	7.7	8.1
8	8.1	8.0	7.9	7.9	8.4
10	7.7	7.8	6.5	7.8	8.5
Average	7.6^{b}	7.6^{b}	7.4^{c}	7.5^{bc}	7.8^{a}
$LSD_{0.05}$	0.14**				

$LSD_{0.05}$ = Least significant difference at P<0.05.

** Means significantly different at P<0.001. Means with different superscripts within row differs significantly at P<0.05.

ABCP- *L. acidophilus*, *B. animalis*, *L. paracasei* subsp. *paracasei*, *P. freudenreichii*

ABC- *L. acidophilus*, *B.animalis*, *L. paracasei* subsp. *paracasei*

BCP- *B. animalis*, *L. paracasei* subsp. *paracasei*, *P. freudenreichii*

BC- *B. animalis*, *L. paracasei* subsp. *paracasei*

C- *L. paracasei* subsp. *paracasei*

Table 4.5 Changes in $\log_{10}$ population (cfu g^{-1}) of *Lactobacillus rhamnosus* in dips with different bacterial combinations over 10 weeks of storage

Storage time (weeks)	Bacterial combination				
	ABRP	ABR	BRP	BR	R (Control)
0 (initial)	7.4	7.4	7.4	7.4	7.4
1	7.5	7.5	7.4	7.3	7.4
2	7.4	7.7	7.5	7.3	7.4
4	7.5	7.4	7.4	7.7	7.6
6	7.5	7.5	7.3	7.2	7.8
8	7.6	7.4	7.3	7.5	8.0
10	7.6	7.0	7.0	7.2	8.1
Average	7.5^{ab}	7.4^{b}	7.3^{c}	7.4^{b}	7.6^{a}
$LSD_{0.05}$	0.15**				

$LSD_{0.05}$ = Least significant difference at P<0.05.

** Means significantly different at P<0.001. Means with different superscripts within row differs significantly at P<0.05.

ABRP- *L. acidophilus, B. animalis, L. rhamnosus, P. freudenreichii*

ABR- *L. acidophilus, B. animalis, L. rhamnosus*

BRP- *B. animalis, L. rhamnosus, P. freudenreichii*

BR- *B. animalis, L. rhamnosus,*

R- *L. rhamnosus*

Table 4.6 Changes in $\log_{10}$ population (cfu g^{-1}) of *Propionibacterium freudenreichii* subsp. *shermanii* in dips with different bacterial combinations over 10 weeks of storage

Storage time (weeks)	Bacterial combination				
	ABCP	ABRP	BCP	BRP	P (Control)
0 (initial)	7.4	7.4	7.4	7.4	7.4
1	<4.0	<4.0	<4.0	<4.0	6.0
2	<4.0	<4.0	<4.0	<4.0	6.1
4	4.3	4.3	<4.0	<4.0	6.5
6	5.6	6.0	6.0	5.4	7.0
8	6.0	6.0	6.1	6.6	7.2
10	6.5	6.5	6.5	6.5	7.3
Average	5.4^b	5.5^b	5.4^b	5.4^b	6.8^a
$LSD_{0.05}$	0.09**				

$LSD_{0.05}$ = Least significant difference at P<0.05.

** Means significantly different at P<0.001. Means with different superscripts within row differs significantly at P<0.05.

ABCP- *L. acidophilus*, *B. animalis*, *L. paracasei* subsp. *paracasei*, *P. freudenreichii*

ABRP- *L. acidophilus*, *B. animalis*, *L. rhamnosus*, *P. freudenreichii*

BCP- *B. animalis*, *L. paracasei* subsp. *paracasei*, *P. freudenreichii*

BRP- *B. animalis*, *L. rhamnosus*, *P. freudenreichii*

P- *P. freudenreichii*

Table 4.7 Ranks of survival rate of *Lactobacillus acidophilus*, A; *Bifidobacterium animalis*, B; *Lactobacillus paracasei* subsp. *paracasei*, C and *Propionibacterium freudenreichii* subsp. *shermanii*, P in different probiotic combinations

Bacteria	Rank of bacterial combination for each type of bacteria							
	ABCP	ABRP	ABC	ABR	BCP	BRP	BC	BR
A	2	3	1	4				
B	1	2	2	2	4	3	3	4
C	2		1		4		3	
R		1		2		4		3
C/R	2	3	1	4	7	8	5	6
P	1	1			1	1		

ABCP- *L. acidophilus*, *B. animalis*, *L. paracasei* subsp. *paracasei*, *P. freudenreichii*
ABRP- *L. acidophilus*, *B. animalis*, *L. rhamnosus*, *P. freudenreichii*
ABC- *L. acidophilus*, *B. animalis*, *L. paracasei* subsp. *paracasei*
ABR- *L. acidophilus*, *B. animalis*, *L. rhamnosus*
BCP- *B. animalis*, *L. paracasei* subsp. *paracasei*, *P. freudenreichii*
BRP- *B. animalis*, *L. rhamnosus*, *P. freudenreichii*
BC- *B. animalis*, *L. paracasei* subsp. *paracasei*
BR- *B. animalis*, *L. rhamnosus*

Table 4.8 Effect of acid type and initial pH on log 10 population (CFU g^{-1}) of *Lactobacillus acidophilus*, *Bifidobacterium animalis*, *Lactobacillus paracasei* subsp. *paracasei* and *Propionibacterium freudenreichii* subsp. *shermanii* over 10 weeks of storage

Time (weeks)	Organic acid type/ Initial pH									
	Lactic acid			Citric acid			Acetic acid			Control (all 3 acids)
	4.45	4.30	4.20	4.45	4.30	4.20	4.45	4.30	4.20	4.45
Lactobacillus acidophilus										
0	6.4	6.4	6.4	6.4	6.4	6.4	6.4	6.4	6.4	6.4
2	6.0	5.7	5.5	5.7	5.5	5.3	5.2	5.2	5.1	5.9
4	5.2	5.2	5.1	5.3	5.1	5.1	5.2	5.0	5.0	5.3
6	5.3	5.2	5.2	5.3	5.1	5.2	5.2	5.1	5.1	5.5
8	5.3	5.2	5.2	5.4	5.2	5.1	5.1	5.1	5.1	5.8
10	5.5	5.1	5.2	5.3	5.2	4.4	5.1	4.9	4.0	5.5
Average	*5.6*	*5.5*	*5.4*	*5.6*	*5.4*	*5.3*	*5.4*	*5.3*	*5.1*	*5.7*
Bifidobacterium animalis										
0	6.4	6.4	6.4	6.4	6.4	6.4	6.4	6.4	6.4	6.4
2	5.0	5.2	5.3	5.2	5.3	5.1	5.2	5.3	5.2	5.5
4	5.1	4.7	4.8	5.2	5.1	4.8	5.0	5.3	5.1	5.3
6	5.3	4.3	4.7	5.1	5.5	5.0	5.0	5.5	5.3	5.2
8	5.2	4.5	4.6	5.1	5.0	5.5	5.2	5.0	5.1	5.1
10	4.2	4.6	4.7	4.4	4.8	4.6	4.4	4.1	4.0	5.5
Average	*5.2*	*5.0*	*5.1*	*5.2*	*5.4*	*5.2*	*5.2*	*5.3*	*5.2*	*5.5*
Lactobacillus paracasei subsp. *paracasei*										
0	6.4	6.4	6.4	6.4	6.4	6.4	6.4	6.4	6.4	6.4
2	6.6	6.4	6.3	6.4	6.5	6.4	6.4	6.2	6.2	6.8
4	6.5	6.2	6.3	6.2	6.3	6.4	6.0	6.0	6.0	6.5
6	6.4	6.1	6.2	6.1	6.2	6.1	5.9	6.2	5.7	6.4
8	6.4	6.1	6.2	6.1	6.0	6.1	5.8	6.2	6.0	6.4
10	6.4	6.1	6.0	5.7	5.9	6.9	5.5	5.5	6.7	6.2
Average	*6.4*	*6.2*	*6.3*	*6.2*	*6.2*	*6.4*	*6.0*	*6.1*	*6.2*	*6.4*
Propionibacterium freudenreichii subsp. *shermanii*										
0	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5
2	5.4	5.3	5.5	5.5	5.8	5.5	5.8	6.0	5.0	5.8
4	7.0	6.8	7.0	7.1	7.0	7.1	7.1	7.1	7.0	6.9
6	7.1	7.0	7.0	7.1	7.0	7.1	7.1	7.1	7.2	7.0
8	7.1	7.1	7.1	7.2	7.2	7.1	7.2	7.2	7.1	7.3
10	6.9	6.9	6.9	7.0	6.8	6.8	7.0	6.9	6.9	7.1
Average	*6.8*	*6.7*	*6.8*	*6.9*	*6.9*	*6.9*	*6.9*	*7.0*	*6.8*	*6.9*

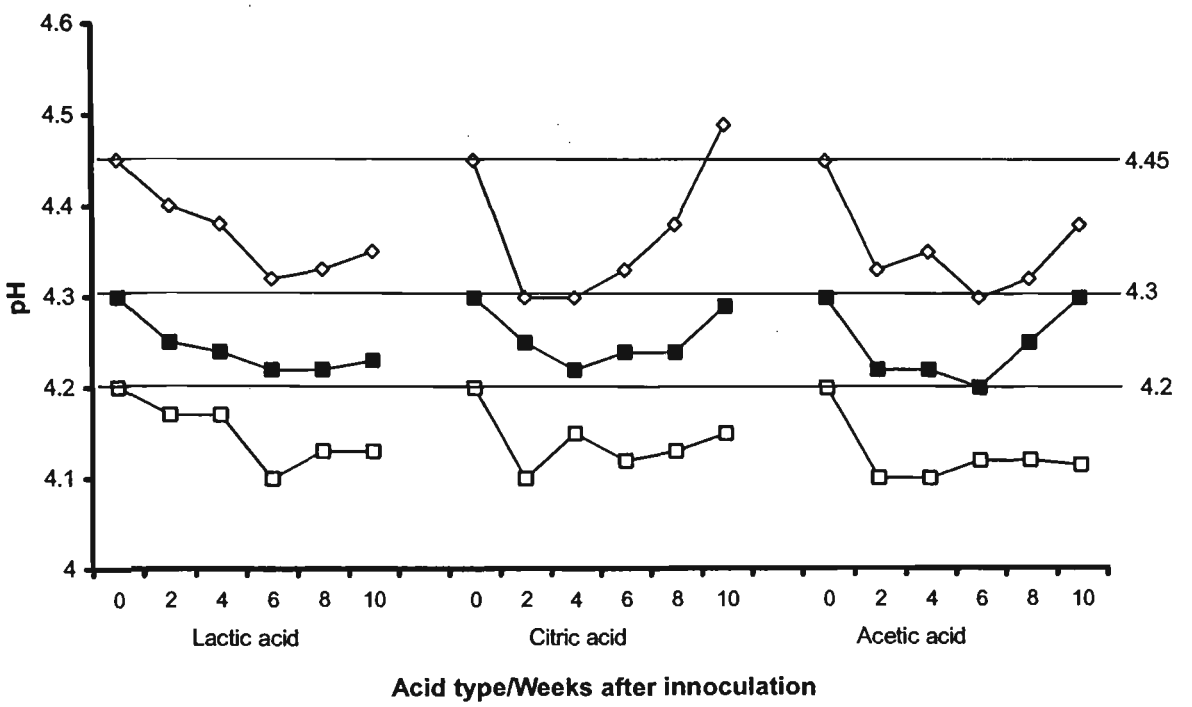

Figure 4.1 Changes in the pH of dips made with different acids (lactic, acetic or citric acid) with different initial pH (4.45, 4.30, and 4.20), over a period of 10 weeks

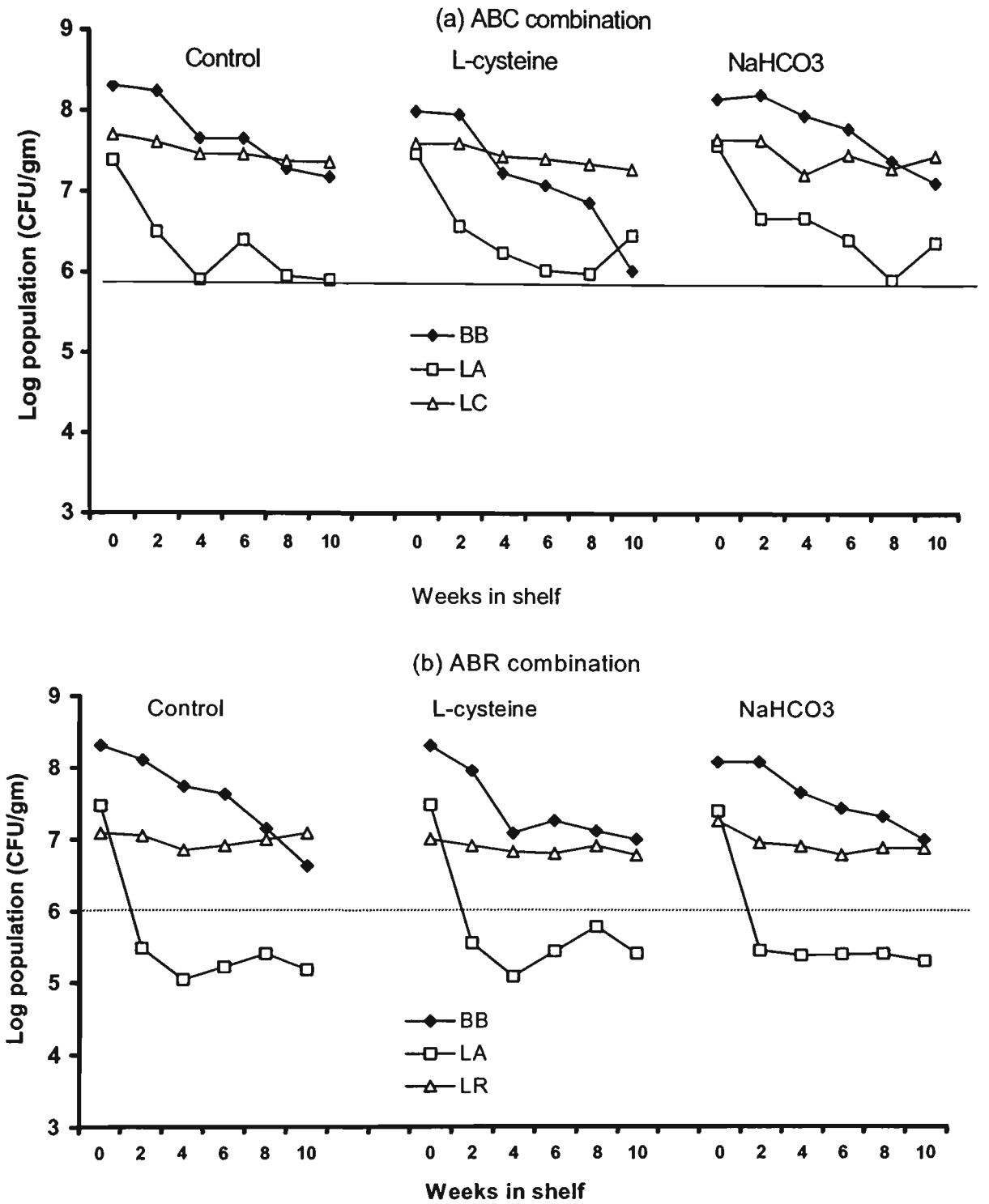

Figure 4.2 Effect of addition of L-cysteine, $NaHCO_3$ to French onion dip and bacterial combinations either *Lactobacillus acidophilus*; LA, *Bifidobacterium animalis*; BB and *Lactobacillus casei*; LC (ABC; top) or *Lactobacillus acidophilus*; LA, *Bifidobacterium animalis*; BB and *Lactobacillus casei* subsp. *rhamnosus*; LR (ABR; bottom) on log population of LA, BB and LC or LR over a period of 10 weeks. 'Control' treatment did not have any chemicals.

CHAPTER 5

ANTIMICROBIAL EFFECTS OF *LACTOBACILLUS ACIDOPHILLUS, LACTOBACILLUS PARACASEI* SUBSP. *PARACASEI, LACTOBACILLUS RHAMNOSUS, BIFIDOBACTERIA* AND *PROPIONIBACTERIA*

5.1. Introduction

5.1.1. Bio-preservation of foods

Preservation of food by biological methods has originated with the use of fermentation to produce wine, vinegar, yoghurt, cheese, butter and bread long before biblical age when civilization entered into the metal age. Bio-preservatives are anti-microbial compounds that are of plant, animal or microbial origin that do not have any adverse effect on human health. Fermented foods are good example of bio-preserved foods in which the starter cultures are allowed to grow in order to produce anti-microbial metabolites.

5.1.2 Current status of bio-preservation

The busy life style in developed world demands convenient food that does not require extensive preparation. Consumers are increasingly concerned about the loss of nutritional value of harshly processed foods and the possible health risk of foods preserved with certain chemical preservatives. Consumer interests in foods that are natural, fresh and healthy are increasing. These include minimally processed meats, vegetables, salads, pastas, dips, sauces and other side dishes, what is known as the 'new generation of refrigerated or chilled foods'. These foods contain minimal amounts or no preservatives at all and are relatively fresh, nutritious and close to natural as compared to most fast and frozen foods. Despite improved manufacturing conditions and implementation of effective legislative control on food processing procedures, such as hazard analysis and critical control points (HACCP) in the food industries, the number of food borne illness still remains a concern in the food industry. This is mainly due to the psychrotrophic nature of some pathogens and the overall risk of contamination and growth of these psychrotrophic pathogenic and spoilage microorganisms in refrigerated foods such as dips. These microorganisms include several groups of bacteria, yeasts and molds. To

minimize the health hazard associated with contamination of food, regulatory agencies and food industries have set up standards or specifications for the tolerance level of pathogens in foods. Table 5.1 shows the minimum growth conditions for the common psychrotrophic pathogenic and spoilage microorganisms. Ready-to-eat foods should have a "zero tolerance" level for *Clostridium botulinum, Escherichia coli, Salmonella* spp. and *Listeria monocytogenes*, but may contain low levels (less than100/g) of *Staphylococcus aureus* (Food Safety Australia, 2001). The use of naturally produced anti-microbial agents, that has no adverse effects on human health such as bacteriocins, to inhibit the proliferation of pathogenic microorganisms in food is a more congenial option to overcome the problems associated with food contamination.

5.1.3. Inhibitory effects of probiotic and lactic acid bacteria on spoilage and pathogenic bacteria

Nisen-Meyer and Nes (1997) suggested that, to maintain their existence or ecological niche, many bacterial species have developed an anti-microbial defense system against competitors. Microorganisms of genera *Lactococcus*, *Lactobacillus*, *Leuconostoc*, *Streptococcus*, *Pediococcus, Enterococcus* and *Carnobacterium*, probiotic bacteria that are of human origin such as *L. acidophillus*, *L. casei, L. rhamnosus, Bifidobacterium* spp. and dairy strains of *Propionibacterium* are reported to produce anti-microbial compounds (Conway, 1996; Daly and Davis, 1998). Organic acids, short chain fatty acids, hydrogen peroxide, reuterin, diacetyl, bacteriocins and bacteriocin-like inhibitory substances are some of the metabolic products of these bacteria are suggested to have potential antimicrobial effects (Holzapfel *et al.*, 1995; Ouwehand, 1998; Ray and Daeschel, 1992; Cleveland *et al.*, 2001; Shah and Dave, 2002). Inhibition by anti-microbial metabolites, competition for nutrition and niche and altered redox potential are some of the ways in which the pathogenic and spoilage organisms are inhibited. Many anti-microbial agents have been in use for a long time without any known adverse effects. For example, many of the organic compounds used in the food industry are also antimicrobial metabolites of bacteria associated with fermented food products. Lactic acid produced by the starter culture in yoghurt prevents the growth of undesirable microorganisms (Ray and Daeschel, 1992).

Organic acids such as lactic and acetic acids produced by lactic acid bacteria help to lower the pH and create unfavourable environment for other organisms. For many years, the hydrogen ion was believed to be associated with the antimicrobial effect. However, recently, bacteriostatic and bactericidal effects of these weak acids are found to be caused by the un-dissociated molecules of these acids, rather than the hydrogen ion. The un-dissociated acid molecules are found to damage the pathogens through acidification of cytoplasm, destruction of the trans-membrane proton motive force, loss of active transport of nutrient through the membrane and by causing sub-lethal injury (Booth and Kroll, 1989; Brown and Booth, 1990; Kabara and Eklund, 1990; Shah and Dave, 2002). The concentration, pH, pKa, lipophilic property and solubility of the acids, the micro-environmental temperature and the microbial load of the media influence the antimicrobial effect of the acid (Brown and Booth, 1990; Kabara and Eklund, 1990). Some strains of LAB including Lactococci, Lactobacilli, Leuconostocs, and Pediococci have the ability to produce hydrogen peroxide but do not catalyze it, thereby acquiring a protection by the accumulated hydrogen peroxide in the growth media (Shah and Dave, 2002). These authors further stated that hydrogen peroxide inhibits the growth of *S. aureus, E. coli, Salmonella typhimurium, Clostridium perfringens, Pseudomonas* spp. and other psychrotrophs. *L. reuteri* (previously classified as *L. fermentum*) produces reuterin during glycerol metabolism. Reuterin is active against a broad spectrum of Gram positive and Gram – negative bacteria (Talarico *et al.*, 1988; Axelson *et al.,* 1989; Chung *et al.*, 1989; Nakanishi, 2002) and fungi (Magnusson and Schnurer, 2001). Most lactic acid bacteria produce diacetyl (2,3-butanedione) during the stationary growth phase by metabolizing the pyruvate accumulated during the exponential growth phase. Some citrate fermenting bacteria such as *Lactococcus lactis* subsp. *lactis* var. *diacetylactis* and *Leuconostoc* spp. produce diacetyl through the fermentation of citrates. Diacetyl shows a broad-spectrum anti-microbial activity against Gram negative and Gram positive bacteria and yeast and mould (Ray and Daeschel, 1992). Dieleveux *et al.* (1998) attributed phenyllactic acid to the inhibition of various pathogenic bacteria such as *L. monocytogenes, S. aureus, E. coli* and *Aeromonas hydrophila*. Above all, phenyllactic acid has been reported to be one of the most abundant aromatic acids to which anti-

microbial properties have been attributed and occur in several honeys with different geographical origins (Steeg and Montag 1987; Weson *et al.,* 1999).

5.1.4. Inhibitory effects of probiotic and lactic acid bacteria on yeast and mould

Yeasts and moulds cause health hazard from myco-toxins and considerable spoilage of food. It is estimated that between 5-10% of world's food production is lost due to fungal deterioration (Pitt and Hocking, 1999). *Aspergillus, Penicillium* and *Fusarium* species have been reported as spoilage organisms during storage of a wide range of food products (Filtenborg *et al.,* 1996; Samson *et al.,* 2000). *Penicillium roqueforti* commonly spoil cheese and cheese based food products. Many strains of yeast are important spoilage organisms of yoghurt and other fermented food products (Pitt and Hocking, 1999).

Many organic acids are used in the food industry to control spoilage organisms (Brul and Coote, 1999). Benzoates, sorbates and propionic acid are primarily used as anti-fungal agents (Davidson, 2001). Natamycin, an antibiotic agent is very effective against yeast and mould, and often used as anti-fungal surface application in foods (Davidson, 2001).

Microorganisms including yeast and mould are becoming resistant to preservatives (benzoates and sorbates) and antibiotics (natamycin) (Brul and Coote, 1999; Loureiro, 2000; Viljoen, 2001; Sanglard, 2002). Davidson (2001) reported that a number of *Penicillium, Saccharomyces* and *Zygosaccharomyces* spp. can grow in the presence of potassium sorbate and degrade it. *P. roqueforti* has been found to be resistant to benzoate (Nielson and Dboer, 2000). The mould *P. discolor* is found to have acquired resistance to natamycin even at a very high concentration (Filtenborg *et al.,* 1996; Nielson and Dboer, 2000). Thus alternative means are needed to control these organisms.

LAB are known to produce anti-microbial substances mainly in the form of organic acids and bacteriocins. Very few reports have been published about the production of specific anti-fungal substances from LAB especially from the probiotic bacteria.

El-Gendy *et al.* (1981) reported that a strain of *L. casei* inhibited growth and aflatoxin production of *Aspergillus parasiticus*. Suzuki *et al.* (1991) have reported anti-

fungal activity of *Leuconostoc mesenteroids* strain from cheese. Bread spoilage moulds such as *Fusarium*, *Penicillium*, and *Aspergillus* are found to be inhibited by *L. sanfrancisco* CBI, on isolate from sourdough (Magnusson and Schnürer 2001).

Vandenberg (1993) reported production of a proteinaceous anti-fungal agent by *L. casei* subsp. *rhamnosus*. Anti-fungal peptides produced by *L. coryneformis* subsp. *coryneformis* (Magnusson and Schnurer, 2001) and *L. pentosus* (Okkers *et al.*, 1999) also have been reported.

Rocken (1996) attributed the production of acetic acid to the anti-fungal activity observed in sourdough. Lavermicocca *et al.* (2000) found that this effect was due to phenyllactic acid and 4-hydroxyphenyllactic acid produced by *L. plantarum* together with lactic and acetic acids. This bacterium was also found to produce anti-fungal low molecular weight substances such as benzoic acid, methylhydantoin, mevalonolacton, and anti-fungal cyclic peptides (Niku-Paavola *et al.*, 1999; Ström *et al.*, 2002). Fungi-static bacteriocin like substance pentocin TV35b was isolated from *L. pentosus* strain (Okkers *et al.*, 1999).

Short chain fatty acids in particular caproic acid produced by *L. sanfrancisco* CBI was found to be the inhibitory substances on sourdough bread spoilage molds such as *Fusarium*, *Penicillium*, and *Aspergillus* spp. (Magnusson and Schnürer 2001).

The aim of the study was to identify the anti-microbial substances produced by selected probiotic bacteria against pathogenic and spoilage bacteria and yeasts and moulds.

5.2. Materials and Methods

5.2.1. Microorganisms and their maintenance

5.2.1.1. Probiotic bacterial cultures

Cultures of *Lactobacillus acidophilus* (LAC1), *Bifidobacterium animalis* (BLC1), *Lactobacillus paracasei* subsp. *paracasei* (LCS1) and *Propionibacterium freudenreichii* subsp. *shermanii* (Pb10360) were obtained from DSM (DSM Food Specialties, Australia Pty. Ltd., Werribee, Australia). *L. acidophilus* (LA5), *B. animalis* (Bb12), *L. paracasei* subsp. *paracasei* (LC01) and *P. freudenreichii* subsp. *shermanii* (PS1) were received from Chr. Hansen (Chr. Hansen Pty. Ltd. Bayswater, Australia). *L. acidophilus* (LA-74),

B. animalis (BF-420) and *L. rhamnosus* (LC 705) were obtained from Danisco Cultor (Danisco Australia Pty. Ltd., Moorabbin, Australia). *L. rhamnosus* LBA was obtained from Rhodia (Rhodia Australia Pty. Ltd., Nottinghill, Australia) and *P. freudenreichii* subsp *shermanii* P was obtained from Bronson and Jocobs Pty. Ltd. (Bronson and Jocobs Pty. Ltd., Dingley, Melbourne, Australia). *L. rhamnosus* strain LR1254 was received from Culture Collection of Victoria University of Technology (Werribee, Australia). Before use, all organisms were tested for purity using Gram stain and sugar fermentation pattern. Yakult drink and Valiaa yoghurt were purchased from the supermarket. *L. casei* Shirota strain (YLC) and *L. rhamnosus* (LGG) were isolated from Yakult drink and Valiaa yoghurt using the method described by Tharmaraj and Shah (2003) and grown in RSM and stored at –37°C. The starter cultures other than YLC and LGG were in freeze-dried direct vat set (DVS) or frozen DVS form. The storage and maintenance of the cultures were carried out as per the recommendation of the manufacturers.

5.2.1.2. Pathogenic and spoilage organisms

Pathogenic organisms including *E. coli, S. typhimurium, S. aureus, Bacillus cereus, Candida albicans* and spoilage organisms such as *B. sterothermophilus, A. niger, P. roqueforti, Fusarium* spp. and *Saccaromyces cerevisiae* were obtained from Victoria University Culture Collection (Werribee, Australia).

5.2.2. Culture media and incubation conditions

Bacterial cultures were maintained at -37°C. *L. acidophilus*, *B. animalis, L. casei, L. paracasei* subsp. *paracasei* and *L. rhamnosus* were grown in 11% non-fat dry milk supplemented with 1% glucose and 0.3% yeast extract (RSM). L-cysteine hydrochloride (0.05%) was added for growing *B. animalis*. *P. freudenreichii* subsp. *shermanii* was grown in sodium lactate broth (NaLa broth) (Tharmaraj and Shah, 2003). Pathogenic and spoilage organisms were maintained in filter sterilized glycerol-nutrient broth. The overnight grown cultures were then transferred into 2 ml cryogenic vials (Iwaki Glass, Canada) and stored at -37°C. Working cultures were made from the frozen stock cultures. To make working cultures, 1 ml of frozen cultures was inoculated in 9 ml aliquots of suitable broth media such as MRS broth for *L. acidophilus*, *L. casei*, *L. paracasei* subsp.

paracasei and *L. rhamnosus,* MRS broth + L cysteine hydrochloride (0.05%) for *B. animalis*, and NaLa broth for *P. freudenreichii* subsp. *shermanii.* Incubation was carried out anaerobically at 37°C for 18 h. for all cultures except for *P. freudenreichii* subsp. *shermanii*, which was incubated for 24 h at 30°C. MRS agar was used for LAB and *B. animalis*, NaLa agar was used for *P. freudenreichii* subsp. *shermanii,* nutrient agar for pathogenic bacteria and OGYE agar (Oxoid, without antibiotics) for yeast and mould, unless otherwise stated. The plates were incubated anaerobically for 48 h at 37°C for *L. acidophilus*, *B. animalis*, *L. casei*, *L. paracasei* subsp. *paracasei* and *L. rhamnosus*. Aerobic and anaerobic incubation for 48 h at 37°C was carried out for spoilage bacteria, anaerobic incubation at 33°C for 72 h for *P. freudenreichii* subsp. *shermanii* and aerobic incubation at 25-30°C for 48-72 h for yeast and mould.

5.2.3. Determination of anti-microbial activity

Spot-on lawn technique described by Tagg *et al.* (1976) was used with some modification for the preliminary detection and screening of inhibitory activity produced by selected probiotic bacteria. The diameter (mm) of the well and the surrounding zones of inhibition were measured. A 0.8% agar media was used to increase the migration of inhibitory substances, instead of 1% agar suggested (Tagg *et al.,* 1976). Twenty-five milliliters of 0.8% suitable agar medium was poured into sterile petri-plates. Wells were cut in the solidified agar using a sterile metal borer (7.0 mm diameter), and the bottom of the wells was sealed with 0.8% agar. Fifty micro-liters of an active culture of producing organisms (strains of *L. acidophilus*, *B. animalis*, *L. casei*, *L. paracasei* subsp. *paracasei*, *L. rhamnosus* and *P. freudenreichii* subsp. *shermanii*) were then filled into the wells. The plates were left at room temperature for 2 h to allow migration and settling of the test cultures, and then incubated for three hours at 37°C. After the initial growth, the remaining depth of the well was sealed with 1% agar. Finally the spotted plates were overlaid with ~ 10 ml of 0.8% agar seeded with 1% indicator organisms (approximately $1x10^7$ cfu.mL^{-1} of the probiotic organisms, pathogenic bacteria and yeast and $1x10^4$cfu.mL^{-1} of the mould) and incubated at suitable incubation conditions as mentioned above. After incubation, plates were examined for zones of inhibition and for other effects of suppression around the wells.

The nature of inhibitory substance produced by the organisms in the initial screening was determined in liquid media by the well diffusion technique (Tagg *et al.*, 1976). Suitable agar (0.8%) medium held at 45°C was inoculated with 1% of active culture of the target organism. About 25 ml of the seeded agar was poured in to a sterile Petri dish and wells were cut in the solidified agar as before. Cell-free extract of producer organisms, grown overnight (16-18 h), was collected from active broth of producer organisms by centrifuging (4000 x g, 12min, 4°C) and filter-sterilized the supernatant using 0.45 μm acrodisc (Gelman Sciences, Ann Arbor, MI, USA) membranes. The supernatant was divided into two portions: untreated (A) and nutralised (B) to pH 6 with 5M NaOH. Wells were filled with 200 μL of the above treated and untreated supernatants. The agar plates were then left for 18 h at room temperature for *L. acidophilus*, *B. animalis*, *L. casei*, *L. paracasei* subsp. *paracasei*, *L. rhamnosus* and *P. freudenreichii* subsp. *shermanii* and at 4°C for pathogenic and spoilage bacteria for diffusion of the test material into the inoculated agar. The plates were then incubated anaerobically for *L. acidophilus*, *B. animalis*, *L. casei*, *L. paracasei* subsp. *paracasei*, *L. rhamnosus* and *P. freudenreichii* subsp. *shermanii* using Oxoid anaerobic system BR038B (Unipath Ltd., Hampshire, England) or aerobically for pathogenic and spoilage bacteria.

5.2.3.1. Antagonism among probiotic bacteria

The inhibitory effect of *L. acidophilus*, *B. animalis*, *L. paracasei* subsp. *paracasei*, *L. rhamnosus* and *P. freudenreichii* subsp. *shermanii* against each other was determined. Four strains of *L. acidophilus*, 3 strains of *B. animalis*, 2 strains of *L. paracasei* subsp. *paracasei*, 2 strains of *L. rhamnosus* and 2 strains of *P. freudenreichii* subsp. *shermanii* were selected for this experiment. Spot-on lawn method and well diffusion methods were used in this study. Zones of inhibitions were measured as described earlier.

5.2.3.2. Anti-microbial effect of probiotic bacteria on spoilage and pathogenic bacteria

The inhibitory effect of *L. acidophilus*, *B. animalis*, *L. casei*, *L. paracasei* subsp. *paracasei*, *L. rhamnosus* and *P. freudenreichii* subsp. *shermanii* on pathogenic and spoilage bacteria were studied. Effect of co-culturing probiotic bacteria on pathogenic

and spoilage organisms, spot-on lawn method and well diffusion methods were used in this study. Two strains of *L. acidophilus*, 2 strains of *B. animalis*, 1 strain of *L. casei*, 2 strains of *L. paracasei* subsp. *paracasei*, 4 strains of *L. rhamnosus* and 3 strains of *P. freudenreichii* subsp *shermanii* were used as producer strains. Two Gram negative pathogenic bacteria (*E. coli* and *S. typhimurium*), 1 Gram negative spoilage bacterium, *P. aerouginosa*, 2 Gram positive pathogenic bacteria (*S. aureus* and *B. cereus*), and 1 Gram positive spoilage bacterium (*B. stearothermophilus*) and 2 spore formers (*B. cereus* and *B. stearothermophilus*) were used as indicator organisms. Zones of inhibitions were measured. Finally the effect of probiotic bacteria on pathogenic and spoilage bacteria in dips was determined.

5.2.3.2.1. Bacterial inocula

Before a new working culture was prepared from the frozen stock cultures, all cultures were propagated twice before use, and sub-cultured into suitable broth weekly for a maximum of 10 subcultures. For routine culturing of Lactobailli, *B. animalis*, *P. freudenreichii* subsp. *shermanii* and pathogenic and spoilage bacteria, MRS broth, MRS broth supplemented with 0.05% L-cysteine-hydrochloride, sodium lactate broth and nutrient broth were used, respectively.

5.2.3.2.2. Spot on lawn assay for bacteria

Spot-on lawn assay was used to study the inhibitory effect of *L. acidophilus*, *B. animalis*, *L. casei*, *L. paracasei* subsp. *paracasei*, *L. rhamnosus* and *P. freudenreichii* subsp. *shermanii* on pathogenic and spoilage bacteria. Two strains of *L. acidophilus*, 2 strains of *B. animalis*, 1 strain of *L. casei*, 2 strains of *L. paracasei* subsp. *paracasei*, 4 strains of *L. rhamnosus* and 3 strains of *P. freudenreichii* subsp. *shermanii* were used as producer strains. One strain each of *E. coli*, *S. typhimurium*, *S. aureus*, *B. cereus*, *B. stearothermophilus* and *P. aeroginosa* were used as indicator organisms. Zones of inhibition were measured and recorded.

5.2.3.2.3. Well diffusion assay for bacteria

Well diffusion method was used to identify the nature of inhibitory substances. Since strains of *P. freudenreichii* subsp. *shermanii* did not show significantly notable inhibitory effect, they were not included in the study. Supernatants of two strains of *L. acidophilus*, 2 strains of *B. animalis*, 1 strain of *L. casei*, 2 strains of *L. paracasei* subsp. *paracasei*, and 4 strains of *L. rhamnosus* were tested. One strain each of *E. coli*, *S. typhimurium*, *S. aureus*, *B. cereus*, *B. stearothermophilus* and *P. aeroginosa* were used as indicator organisms. Zones of inhibition were measured and recorded

5.2.3.2.4. Effect of co-culturing probiotic bacteria with pathogenic and spoilage bacteria

This method was used to study the effect of probiotic bacteria on the population of pathogenic and spoilage bacteria in reconstituted skim milk (RSM) medium. A 9 mL aliquot of RSM was inoculated with 1 mL over night culture of probiotic bacteria and 0.1 mL of pathogenic or spoilage bacteria. Inoculated RSM medium was mixed well and incubated at 37° C for 24 h. Following incubation, the population of spoilage and pathogenic bacteria were counted on nutrient agar.

5.2.3.2.5. Effect of probiotic bacteria on the inoculated pathogenic and spoilage bacteria in French onion dip

French onion dip was obtained from the production line of Poseidon and Black Swan Pty. Ltd. (Clayton, Victoria, Australia). Two kilogram lots of dips were inoculated with pathogenic and spoilage bacteria (10^7 cfu g^{-1}) and with probiotic bacteria cultures (10^8 cfu g^{-1}). Inoculated dips were packed in 100 g lots in plastic containers and sealed and stored at 4°C. After 24 h of storage, 10 g of inoculated dip was mixed with 90 ml of 0.015% peptone water and the population of pathogenic and spoilage bacteria were counted on nutrient agar.

5.2.3.2.6. Analysis of probiotic culture supernatant for organic acids

Supernatants of *L. acidophilus*, *B. animalis*, *L. casei*, *L. paracasei* subsp. *paracasei*, *L. rhamnosus* and *P. freudenreichii* subsp. *shermanii* were analysed for the end products of fermentation using high performance liquid chromatography (HPLC; Varian Australia

Pty. Ltd., Mulgrave, Australia). Supernatants of overnight cultures of lactobacilli and bifidobacteria that were grown in MRS broth and *Propionibacterium* that was grown in sodium lactate broth at suitable growth conditions using 1% inoculum were subjected to HPLC analysis using the method described by Dubey and Mistry (1996 a & b). Briefly, 100 µL of 15.8 M HNO_3 and 14.9 mL of 0.009 M H_2SO_4 were added to 1.5 mL of overnight-grown cultures and centrifuged at 4°C at 4000 x g for 15 min using a bench top centrifuge (Sorvall RT7, Newton, CT, USA). The supernatant was filtered using 0.22 µm Millipore filters and 2 ml aliquots were stored at –20°C until analysed. The HPLC system consisted of a Varian 9012 solvent delivery system, Varian 9100 auto-sampler, Varian 9050 variable wavelength UV/V turnable absorbance detector and a 730 data module. An Aminex HPX-87H column (300 mm x 7.8 mm, Bio-Rad Laboratories, Richmond, CA, USA) and a guard column with disposable cartridges H+ (Bio-Rad Laboratories) maintained at 65°C were used for the analysis of organic acid. The degassed mobile phase of 0.009 M H_2SO_4, filtered through a 0.45 µm membrane filter was used at a flow rate of 0.3ml/min. The wavelength of detection was optimised at 220 nm and the sample injection was 50 µL. The standard solutions of acetic acid, lactic acid, formic acid, propionic acid and benzoic acid (Sigma Chemical Co., St. Louis, MO, USA) were prepared in mobile phase solution to establish elution times and standard curves.

5.2.3.3. Anti-microbial effect of probiotic bacteria on yeast and mould

The analysis of the inhibitory effect of *L. acidophilus*, *B. animalis*, *L. casei*, *L. paracasei* subsp. *paracasei*, *L. rhamnosus* and *P. freudenreichii* subsp. *shermanii* on yeast and moulds are described here. Initially the bacteria were screened for the presence of inhibitory effect on yeast and mould, in agar media, using spot-on-lawn assay, well diffusion assay and spot and streak diffusion assay. All bacterial strains showed varying degree of inhibitory effects. Two strains of *L. acidophilus*, 2 strains of *B. animalis*, 1 strain of *L. casei*, 2 strains of *L. paracasei* subsp. *paracasei*, 4 strains of *L. rhamnosus* and 3 strains of *P. freudenreichii* subsp. *shermanii* were then tested in RSM medium and in French onion dip by growing the probiotic bacteria with yeast (*C. albicans* and *S. cerevisiae*) and mould (*A. niger, P. roqueforti* and *Fusarium* spp.) together. The population of yeast and mould was counted after 5 days.

5.2.3.3.1. Fungal inocula

The moulds, *A. niger, P. roqueforti* and *Fusarium* spp. were grown on OGYE agar (Oxoid, without antibiotics) at 25°C for 5-7 days (or until sporulation) and then stored at 5°C. Cylinders of mould cultures were made using a sterile metal borer with 7.0 mm diameter from freshly made mold plate. Inocula containing spores or conidia were prepared by adding 3 cylinders of mould into 10 ml sterile peptone water (0.15% wt/ vol) in a McCartney bottle and by shaking them vigorously. Yeast cell inocula were prepared from washed cultures grown in yeast extract glucose peptone (YGP) broth as still cultures at 30°C for 24 h. Mould spores and yeast concentrations were determined using OGYE agar (Oxoid, without antibiotics).

5.2.3.3.2. Well diffusion assay for yeast and mould

The inhibitory effect of *L. acidophilus, B. animalis, L. casei, L. paracasei* subsp. *paracasei, L. rhamnosus* and *P. freudenreichii* subsp. *shermanii* on yeast and mold was studied using well-diffusion assay. Two strains of *L. acidophilus*, 2 strains of *B. animalis*, 2 strains of 1 strain of *L. casei, L. paracasei* subsp. *paracasei*, 4 strains of *L. rhamnosus* and 3 strains of *P. freudenreichii* subsp. *shermanii* were used as producer organisms. The yeast *C. albicans* and *S. cerevisiae* and moulds *A. niger, P. roqueforti* and *Fusarium* spp. were used as indicator organisms.

5.2.3.3.3. Spot and streak assay for yeast and mould

The inhibitory effect of *L. acidophilus, B. animalis, L. casei, L. paracasei* subsp. *paracasei, L. rhamnosus* and *P. freudenreichii* subsp. *shermanii* on yeast and mould was studied using the streak method described by Magnusson and Secnurer (2001) with a slight modification in adding a spot (10μL) with the streak (spot and streak assay). Two strains of *L. acidophilus*, 2 strains of *B. animalis*, 1 strain of *L. casei*, 2 strains of *L. paracasei* subsp. *paracasei*, 4 strains of *L. rhamnosus* and 3 strains of *P. freudenreichii* subsp. *shermanii* were used as producer strains. The yeast *C. albicans* and *S. cerevisiae* and moulds *A. niger, P. roqueforti* and *Fusarium* spp. were used as indicator organisms. Twenty milliliters of 0.8% MRS agar was poured into petridishes and allowed to set. Two

spots and streaks of producer organisms were made by adding one drop (10μL) of active culture and by drawing 2 cm parallel lines from the spots using the tips. Inoculated agar plates were incubated anaerobically for 48 h at 37°C for *L. acidophilus*, *B. animalis*, *L. casei*, *L. paracasei* subsp. *paracasei* and *L. rhamnosus* and at 30°C for *P. freudenreichii* subsp. *shermanii*. Following incubation, the spots and streaks were sealed and overlaid with 10 ml 0.8% agar, inoculated with the test organism (yeast 10^7 cfu/ml or mold 10^4 cfu/ml). Overlaid plates were then incubated aerobically at 30°C for 24-48 h. The plates were examined for zones of inhibition around the spot and streak. The area of zones were graded as follow: -, no suppression; +, no fungal growth for 5mm around the spot and streak; ++, no fungal growth for 10mm around the spot and streak; +++, no fungal growth for 15mm around the spot and streak; ++++ no fungal growth above 15mm around the spot and streak. Following this experiment, to find out the inhibitory effect was due to either hydrogen peroxide or proteinaceous substances, the agar media for the base and overlay were mixed with catalase or crude protease (both from Sigma, USA) with a final concentration of 0.05-0.1μg mL^{-1}, before inoculation of probiotic bacteria or spores of moulds of *P. roqueforti* and *Fusarium* spp.

5.2.3.3.4. Effect of co-culturing probiotic bacteria on yeast in MRS or NaLa broth media

Nine milliliter aliquots of MRS medium was inoculated with 1 mL of *L. acidophilus*, *B. animalis*, *L. casei*, *L. paracasei* subsp. *paracasei*, *L. rhamnosus* and nine milliliter aliquots of NaLa medium was inoculated with 1 mL of *P. freudenreichii* subsp. *shermanii* and 0.1 mL (10^7 cfu/mL) of overnight-culture of yeast in McCartney bottles. The bottles were then incubated at 30°C for 24 h. Serial dilution was carried out in 0.05% peptone water and the yeast population was counted using 3M petrifilms, after 5 days.

5.2.3.3.5. Effect of co-culturing probiotic bacteria on yeast and mould in RSM media

Ten millilitre aliquots of RSM media were inoculated with mould spores (10^4 cfu/ml) or overnight culture of *C. albicans* (10^8 cfu/ml) and 0.1ml active overnight-cultures of *L. acidophilus*, *B. animalis*, *L. casei*, *L. paracasei* subsp. *paracasei*, *L. rhamnosus* and *P. freudenreichii* subsp. *shermanii* in McCartney bottles. The bottles were then incubated at 30° C for 24 h and stored at 4° C for 2 weeks. Serial dilution was carried out in 0.05%

peptone water and the yeast and mold population was counted after 24-h and 15 d storage. The cultures were serially diluted and transferred to 3M yeast and mould petriflims and the mould colonies were counted after 5 days.

5.2.3.3.6. Effect of probiotic bacteria on inoculated yeast and mould in French onion dip

Two-kilogram lots of French onion dip were inoculated with yeast or mould (10^4 cfu g^{-1}) and with probiotic culture (10^8 cfu g^{-1}). Inoculated dips were packed in 100g lots in plastic containers, sealed and stored at 4° C for 10 weeks. Yeast and mould counts were taken on 3M yeast and mould petrifilms and on OGYE agar (Oxoid, without antibiotics). Two containers with each kind of dips were kept outside at room temperature for 3 days to observe yeast and mould growth.

To examine the effect of probiotic bacteria on yeast and mould in the dips, French onion dip was contaminated individually with strains of yeast and mould, inoculated with two combinations of probiotic bacteria (Combination 1 (*L. acidophilus*, *L. paracasei* subsp. *paracasei*, *B. animalis* and *P. fredenreichii* subsp. *shermanii*), Combination 2 (*L. acidophilus*, *L. rhamnosus*, *B. animalis* and *P. fredenreichii* subsp. *shermanii*)) and stored at 4° C. This was compared with a control treatment that did not include probiotic bacteria.

5.2.3.3.7. Effect of addition of metabolites of probiotics to French Onion Dip contaminated with yeast and mould

Two kilogram lots of French onion dips were inoculated with yeast (10^7 cfu g^{-1}) or mould (10^4 cfu g^{-1}) and 5% of metabolites of probiotic bacteria. The metabolites were obtained by heating overnight-grown probiotic cultures in 60° C water bath for 40 min. Inoculated dips were packed in 100g lots in plastic containers and sealed and stored at 4°C for 10 weeks. Yeast and mould counts were taken on 3M yeast and mould petrifilms and on OGYE agar (Oxoid, without antibiotics).

5.3. Results and discussion

5.3.1. Antagonism among probiotic bacteria

Many combinations of probiotic bacteria are being used in probiotic food products (e.g. yoghurt). However, it is not clear whether all the introduced bacteria survive during storage to supply sufficient number of viable bacterial cells to the consumer and in shelf. In this study, the spot-on-lawn assay was used to identify the bacterial species and strains or the ideal ratio of species/strains that do not antagonize each other thereby affecting the survival of organisms when grown in combination.

Between and within species antagonism was observed among *L. acidophilus, B. animalis, L. paracasei* subsp. *paracasei, L. rhamnosus* and *P. freudenreichii* subsp. *shermanii* (Table 5.2). *L. paracasei* subsp. *paracasei* and *L. rhamnosus* species showed the greatest antagonism (zone of inhibition) to all indicator bacterial species tested. All strains of probiotic bacteria, except strains of bifidobacteria and propionibacteria showed certain level of inhibition of self. The exact reason for this self-inhibition is not kknown. When these bacteria were grown in broth cultures as producers, they actively grew and produced metabolites. But when they were transferred from this liquid media into solid agar media, the growth become less active. This may have made the bacteria susceptible to harsher environments such as the presence of the metabolites of their own. Some of these metabolites may have present in the broth culture that was added on the spot. The earlier finding of the prevalence of *L. paracasei* subsp. *paracasei* and *L. rhamnosus* at higher population levels (Tharmaraj and Shah, 2003) in many different bacterial combinations, can be attributed to this strong inhibitory effect of *L. paracasei* subsp. *paracasei* and *L. rhamnosus*. This may also explain the reduction in *L. acidophilus* and *B. animalis* populations in the presence of *L. paracasei* subsp. *paracasei* and *L. rhamnosus* described in Tharmaraj and Shah (2003). *L. paracasei* subsp. *paracasei* and *L. rhamnosus* also inhibited *P. freudenreichii* subsp. *shermanii*. This explains the drastic reduction in the population of *P. freudenreichii* subsp *shermanii* in combination with *L. paracasei* subsp. *paracasei* and *L. rhamnosus* in dips (Tharmaraj and Shah, 2003). *L. paracasei* subsp. *paracasei* and *L. rhamnosus* also showed antagonism between their own strains. However, *L. acidophilus*, *B. animalis* and *P. freudenreichii* subsp. *shermanii* did not show considerable antagonism against *L. paracasei* subsp. *paracasei* and *L. rhamnosus*.

L. acidophilus inhibited *B. animalis* more than *L. paracasei* and *P. freudenreichii* subsp. *shermanii*. *B. animalis* and *P. freudenreichii* subsp. *shermanii* did not inhibit any of the bacterial species or strains tested, except that *B. animalis* showed moderate inhibition to *L. rhamnosus* strain LC705. This is supported by a drastic reduction in the population of *L. rhamnosus* in a combination of *L. acidophilus, B. animalis* and *L. rhamnosus* strain LC705 in French onion dip during storage (Tharmaraj and Shah, 20043). *B. animalis* suppressed *L. acidophilus* and *L. rhamnosus* in dip when inoculated at a level of 2 log units greater (log 9 cfu g^{-1}) than the latter two. This suggests that the inhibitory effect of *B. animalis* may be dose dependent.

All strains of *L. acidophilus* tested moderately controlled *L. rhamnosus* strain LC705 and *L. paracasei* subsp. *paracasei* strain LC01. *L. acidophilus* strain LAC1 controlled all strains of *L. paracasei* subsp. *paracasei*. Out of all strains of *L. acidophilus* tested, LAC1 showed the greatest resistance to other bacterial strains, followed by LAC4 and LA5. This suggests that, in a cocktail of different strains of probiotics, LAC1 may grow better than the other three strains. All strains of *B. animalis* were affected to similar degrees by all strains of *L. acidophilus*, *L. paracasei* subsp. *paracasei* and *L. rhamnosus*. *L. paracasei* subsp. *paracasei* showed the greatest resistance to all other probiotic bacteria.

This indicates that both between and within species antagonism can affect the survival of probiotic bacteria and care is needed in selecting the ideal probiotic bacterial combination and/or ratio for a food product. *L. acidophilus* strain LAC1, *B. animalis* strain BB12 and *L. paracasei* subsp. *paracasei* strains either LC01or LBC81 were found to be the best ABC combination for a probiotic combination that can survive best in a food application (Table 5.2). When used in combination, *L. paracasei* subsp. *paracasei* and *L. rhamnosus* should be included at a relatively lower ratio (at least 1log less) to *L. acidophilus*, *B. animalis* and *P. freudenreichii* subsp. *Shermanii*, thus the inhibitory effects of all the bacteria can be balanced and allowing all bacteria to survive above the required level.

Well diffusion-assay was used to establish the nature of the inhibitory substance/s produced by the producing bacteria against other bacteria. The supernatant of probiotic bacteria tested in this method did not show any notable zones of inhibition against any of

the probiotic bacteria/strains tested. However, HPLC analysis of the supernatant (Table 5.3) indicated the presence of organic acids (acetic, lactic, formic, propionic butyric benzoic and phenyl lactic acids) in varying quantities. Therefore, the results cannot be conclusively interpreted that there was no inhibitory substances produced, rather the method used here may have not identified and accounted for all the inhibitory substances. The lack of inhibitory activity may either be due to insufficient production of inhibitory substance/s or loss of inhibitory substances through evaporation of volatile inhibitory components such as hydrogen peroxide, short chain fatty acids such as formic acid and propionic acid, aldehydes and alcohols (Daeschel, 1992). Barefoot and Klaenhammer (1983) suggested that part of the active inhibitory substance produced by probiotic bacteria could be absorbed and bound to the cell mass thereby escaping the supernatant during extraction. These authors suggested that some inhibitory substances might be destroyed or may have disappeared at the end of the stationary phase. It may also be possible that some acid-based inhibitory substance/s could have been diluted in the broth culture, disabling it to produce visible zones of inhibition against probiotic bacteria that are considerably resistant to organic acids.

5.3.2. Effect of probiotic bacteria on pathogenic and spoilage bacteria

Antagonism of probiotic bacteria on spoilage and pathogenic bacteria can be affected by many factors including bacterial load, growth conditions and resistance of the pathogen to the inhibitory substances. In this experiment, different broth media (nutrient broth, MRS broth and sodium lactate broth), different growth conditions (aerobic and anaerobic) and different agar media were evaluated using spot-on-lawn assay to assess the anti-microbial properties of the probiotic bacteria.

In a preliminary spot-on-lawn assay, the indicator organisms were tested for antagonistic effects by probiotic bacteria under aerobic conditions. In this experiment, the indicator organisms (*E. coli, S. typhimurium, P. aeroginosa, S. aureus, B. cereus, B. stearothermophilus*) were inoculated individually in nutrient agar and were overlaid on a plain nutrient agar plate (control) or over plates with producer organisms (*L. acidophilus, B. animalis, L. casei, L. paracasei* subsp. *paracasei, L. rhamnosus* or *P. freudenreichii* subsp. *shermanii*) that were grown with nutrient broth. The system was then incubated

aerobically at 37°C. The pathogen and spoilage organisms showed prolific growth on the control plates and on plates with *L. acidophilus* and *B. animalis*. Plates with *L. paracasei* subsp. *paracasei* and *L. rhamnosus* showed limited growth of *E. coli, S. typhimurium*, and *S. aureus*. The poor tolerance of *L. acidophilus* and *B. animalis* to aerobic conditions may have affected their establishment and therefore the ability to show inhibitory effects. This clearly indicates that aerobic condition affects some probiotic bacteria more than the other, in producing inhibitory substances. However, due to their relative ability to tolerate aerobic condition, *L. casei*, *L. paracasei* and *L. rhamnosus* were able to establish and show some inhibitory action against *E. coli, S. typhimurium*, and *S. aureus*. Further, the growth media (nutrient broth) might not have provided enough nutrient (especially sugar) to the probiotic bacteria, thereby affecting their growth and ability to produce sufficient quantity of organic acids that inhibits pathogenic and spoilage bacteria.

When this preliminary experiment was repeated by replacing the producer growth media from nutrient broth to MRS broth, *L. casei*, *L. paracasei* subsp. *paracasei* and *L. rhamnosus* showed clear inhibitory effects against *E. coli, S. typhimurium*, and *S. aureus*. *L. casei*, *L. paracasei* subsp. *paracasei* and *L. rhamnosus* produced clear inhibitory zones against *P. aerouginosa* and slight inhibitory effect against *B. cereus* and *B. stearothermophilus*. This suggests that sugar (in MRS media) may influence the production of inhibitory substances. In this preliminary experiment, *P. freudenreichii* subsp. *shermanii* strain P showed inhibition against *B. stearothermophilus* and on *B. cereus*. The inhibition zone produced against *B. stearothermophilus* and *B. cereus* was very sharp and with a definite margin without a diffusion zone. Though not initially planned in the experiment, the plates were kept in the refrigerator (4°C) for 2 weeks. After 2 weeks of storage in the refrigerator, the zone of inhibition produced on *B. stearothermophilus* did not disappear but that of *B. cereus* started to shrink. The thermophilic nature of *B. stearothermophilus* would have prevented it from growing at 4°C thus not affecting the zone of inhibition. One possible explanation for reduction in the zone of inhibition exhibited by *B. cereus* is that, either the inhibitory substance produced by *P. freudenreichii* subsp. *shermanii* strain P might not be stable at 4°C or *P. freudenreichii* subsp. *shermanii* strain P might not have been active at 4°C. Another

reason may be that the psychrotrophic bacteria, *B. cereus* might have developed resistance to the substance in the absence of its continuing production. This indicates that storage temperature can also affect the anti-microbial effect of probiotics.

When the producer organisms were grown in suitable media (MRS broth for *L. acidophilus, L. casei, L. paracasei* subsp. *paracasei* and *L. rhamnosus*, MRS broth + 0.05% of L-cysteine for *B. animalis* and NaLa broth for *P. freudenreichii* subsp. *shermanii*) with the overlay of indicator organisms (*E. coli, S. typhimurium, P. aeroginosa, S. aureus, B. cereus, B. stearothermophilus*) inoculated in nutrient agar and incubated anaerobically at 37° C, all producer organisms produced inhibitory zones except the aerobic bacteria, *P. aeroginosa* and *B. stearothermophilus* (Table 5.4). *P. aeroginosa* and *B. stearothermophilus* did not grow at all under anaerobic incubation. But, following the anaerobic incubation for 72 h, when incubated aerobically at 37°C for further 24 h, *P. aeroginosa* and *B. stearothermophilus* showed zones of inhibition that were larger than the zones produced on *E. coli, S. typhimurium*, and *S. aureus* and *B. cereus* in the anaerobic phase (Table 5.4). This indicates that these two bacteria are controlled by anaerobic conditions alone and do not require any additional probiotic control under modified or vacuum packed conditions. It is also suggested that the inhibitory substances produced and diffused during the initial anaerobic phase of this experiment may have caused the greater inhibition zones against these two bacteria during the aerobic phase. The proliferation of the probiotic bacteria during the anaerobic phase in the absence of competition from an actively growing indicator organism may also have contributed to the greater inhibitory effect during the aerobic phase.

Under anaerobic conditions, all producer organisms have produced considerable inhibition zones against all pathogenic bacteria (Table 5.4). On an average, among all the probiotic and spoilage bacterial interactions, the spore-formers were inhibited by the probiotics to a greater extent (average zone of inhibition, 19 mm) than the non-spore formers (average zone of inhibition, 14 mm). Also, the Gram positive bacteria were inhibited more (average zone of inhibition, 18 mm) than the Gram negative bacteria (average zone of inhibition, 14 mm). However, this discriminatory inhibition between Gram positive and Gram negative bacteria was not prominent with *B. animalis* (average

zone of inhibition, 15.7 mm). *P. freudenreichii* subsp. *shermanii* inhibited only the Gram positive bacteria (*S. aureus* and *B. stearothermophilus*).

The order of probiotic bacteria in terms of level of inhibition (and zone of inhibition in mm) was *L. rhamnosus* (21)> *L. acidophilus* (19) = *L. casei* (19) > *L. paracasei* subsp. *paracasei* (18) > *B. animalis* (15) > *P. freudenreichi* subsp. *shermanii* (11). There was a considerable difference between probiotic strains in the ability to inhibit pathogenic and spoilage bacteria. Out of the *L. acidophillus* strains, LAC1 inhibited both Gram positive and Gram negative bacteria better than LA5. The strain BB12 was better for both types of pathogenic bacteria among the *B. animalis* strains tested. The *L. casei* strain YLC was the best among the 3, *L. casei* and *L. paracasei* subsp. *paracasei* strains tested followed by *L. paracasei* subsp. *paracasei* strain LCS1. Out of the *L. rhamnosus* strains, LR1524 and GG were found to be better for both types of pathogenic and spoilage bacteria (Table 5.4).

The effect of co-culturing probiotic bacteria with pathogenic and spoilage bacteria in RSM media is shown in Table 5.5. Compared to the control (without any probiotic bacteria) treatment, all four pathogenic and spoilage bacteria were inhibited by all probiotic strains tested to varying degrees. On average, the probiotic bacteria have reduced the population of spoilage and pathogenic bacteria by 2.8 log units, a level that was less than that found in control. *B. cereus* was inhibited to a greater degree by all probiotic bacteria and strains than other pathogenic bacteria. On average, the inhibitory effect of all probiotic bacteria and strains was the weakest against *E. coli*. *S. aureus* was inhibited to a greater degree by *B. animalis* and *L. rhamnosus* than the other probiotic bacteria. The level of reduction in the population of pathogenic bacteria by probiotic bacteria was greatest on *B. cereus* (by 3.6 log units), followed by *S. typhimurium* (by 3.2 log units), *S. aureus* (2.6 units) and *E. coli* (by 1.6 units). *P. freudenreichii* subsp. *shermanii* strain P and *L. acidophilus* showed considerable inhibition against *B. cereus* but not against any other pathogenic bacteria. The results (Table 5.5) indicate that, out of the probiotic bacteria tested, *B. animalis* and *L. rhamnosus* were the most effective against pathogenic bacteria. Results of a well-diffusion assay, conducted to identify the nature of the inhibitory substance are discussed next.

Table 5.6 shows the zone of inhibition (with or without the zone of diffusion) using well-diffusion assay. In general, these results were similar to those found in spot-on-lawn assay, described earlier and in Table 5.4. All probiotic bacterial strains produced a clear zone of inhibition and a less clear zone of diffusion, except *L. rhamnosus* strains LC705 and LBA, which did not show a zone of diffusion. This suggests that part of the inhibitory substance/s produced by these strains may have disappeared or easily destroyed during extraction of the supernatant or produced lesser quantity of anti-microbial compounds that can diffuse. HPLC analysis (Table 5.3) shows that the production of acetic acid is the lowest in these 2 bacteria compared to the other probiotic bacteria. However, *L. rhamnosus* strain GG showed a zone of diffusion. *L. rhamnosus* strain GG produced formic acid as indicated by HPLC analysis (Table 5.3). The reduced quantity of acetic acid could explain the lack of zone of diffusion. *L. rhamnosus* strains LC705 and LBA also might have produced formic acid in lower quantities, which could have been lost during the extraction procedure. These bacteria also could have produced a bacteriocin to aid the acid effect. To decide whether the inhibitory effect were due to acid or any other substances, part of the supernatant was nutralised to pH 6.0 and used. The nutralised supernatant did not produce any zone of inhibition, indicating that the inhibitory substance was a single or a group of organic acids and/or acid derivatives and/or a bacteriocin that is not active at pH 6. The type and mode of action of acids and the results of HPLC analysis conducted to identify the organic acids produced by the probiotic bacteria are discussed in the following paragraph.

Organic acids such as formic acid, acetic acid, lactic acid, propionic acid, benzoic acid and free fatty acids are produced from sugars- (Ray and Sandine, 2000), amino acid-(Gummalla and Broadbent, 2001) and/or lipid-(Magnusson, 2003) metabolism in bacterial cells. These acids that are responsible for the inhibition of pathogenic and spoilage organisms, can be produced by *L. acidophilus, B. animalis, L. casei, L. paracasei* subsp. *paracasei, L. rhamnosus* and *P. freudenreichii* subsp. *shermanii* at varying quantities (Eklund, 1989). Lactic acid bacteria are found to produce large quantities of lactic acid, which reduces the pH of the media (Eklund, 1989). These organic acids inhibit pathogenic organisms by reducing the pH of the environment to hostile levels for other microorganisms. In addition to the pH effect, there are other

modes through which the acids inhibit pathogenic and spoilage microorganisms. Un-dissociated form of weak organic acids diffuse through the pathogenic bacterial cell membrane. These diffused acids dissociate inside the cell to a degree depending on the intracellular pH. The H^+ ions released during the dissociation acidify the cytoplasm to cause collapse of the electrochemical proton gradient, resulting in bacteriostasis and eventual death of the susceptible bacteria (Axelsson, 1998; Piard and Desmazeaud, 1991; Eklund, 1989). When large proportion of the acid is in un-dissociated form, at a pH value that is below the pK_a value of the organic acids, the inhibitory effect is more pronounced (Axelsson, 1998; Piard and Desmazeaud, 1991). The Pk_a values of formic acid, acetic acid, lactic acid, propionic acid, benzoic acid and phenyllactic acid are 3.75, 4.76, 3.86, 4.87, 4.20 and 3.46, respectively. The smaller molecular structure and the lipophilic characteristics of organic acids can also contribute to the anti-microbial action. Lipophilic, smaller un-dessociated molecules can diffuse faster into the cell to effect more damage. Meat Net Newsletter (September 2003) stated that formic acid had the best bactericidal effect on pathogenic bacteria and though the effectiveness depended on the pH, the un-dissociated form of formic acid was the strongest inhibitor compared to acetic acid, propionic acid and hydrochloric acid (Dibner and Buttin, 2002). Dibner and Buttin (2002) also found that at pH 4.0, formic acid reduced the *E. coli* population by 4 log units while lactic acid and hydrochloric acid reduced only by 1.5 log and 0.5 log units, respectively. The reported fungicidal activity of phenyllactic acid (Lavermicocca *et al.*, 2002) against many mould strains including *P. roqueforti* and *Aspergillus flavus*, appear to be related to the lipophilic properties (Gould, 1996). The proportion of organic acids produced by each bacteria on the overnight-cultured supernatants of the probiotic bacteria using HPLC analysis are given in Table 5.3. All the bacteria tested produced large quantities of lactic acid except *Propionibacterium*. On an average, the strains of *L. acidophilus, B. animalis, L. casei, L. paracasei* subsp. *paracasei, L. rhamnosus* and *P. freudenreichii* subsp. *shermanii* produced 103, 57, 106, 97, 94 and 23 mM lactic acid and 46.00, 53.70, 26.20, 28.75, 27.60 and 36.80 mM acetic acid, 2.7, 1.0, 0.7, 0.5, 0.8 and 2.3 mM L^{-1} benzoic acid and 1.35, 0.97, 0.92, 1.08, 2.50 and 0.50 mM of phenyl lactic acid respectively. Except *L. acidophilus* all other strains were found to produce smaller quantities of butyric acid. *P. freudenreichii* subsp. *shermanii* produced around 29.0 mM

propionic acid. *B. animalis*, *P. freudenreichii* subsp. *shermanii* and *L. rhamnosus* strain GG produced formic acid (9.8, 22.8,1.5 mM). The ability to reduce pH by producing large quantities of lactic acid or acetic acid along with the ability to produce formic acid and, perhaps in larger quantities in more favourable conditions, may be attributed to the ability of these bacteria to inhibit pathogenic bacteria. The findings of Dibner and Buttin (2002) were in agreement with this. *L. acidophilus* and *P. freudenreichii* subsp. *shermanii* produced relatively large quantities of benzoic acid while *L. casei*, *L. paracasei* subsp. *paracasei* and *L. rhamnosus* produced very little benzoic acid. All of the strains tested produced considerable amounts of phenyl lactic acid. But *L. acidophilus* strain LAC1 (2.03 mM) and *L. rhamnosus* strain LR1524 (4.14 mM) produced the largest quantities of phenyl lactic acid. Though, strains of *P. freudenreichii* subsp. *shermanii* had the ability to produce all 7 organic acids (Table 5.3), which are potential microbial inhibitors, they did not show inhibition of spoilage and pathogenic bacteria using spot-on-lawn assay (Table 5.4). This can be attributed to higher pH of the media (5.8). The inability of *P. freudenreichii* subsp. *shermanii* to produce large quantities of lactic acid may have contributed to the higher media pH. However, *P. freudenreichii* subsp. *shermanii* strain P inhibited *B. cereus* (Table 5.4), despite a minimum pH requirement of 4.3 for *B. cereus* (Table 5.1). It can be assumed that *P. freudenreichii* subsp. *shermanii* strain P might have produced some other inhibitory substance/s that could be active at higher pH.

Table 5.7 shows the effect of co-culturing probiotic bacteria with French onion dip, on the log population of pathogenic and spoilage bacteria. There had been a reduction of the bacterial population (From log 8 to log 5), in the control treatment. This may have been caused by the acidity of the product (pH 4.3). Though, the probiotic strains have also reduced the log population of the pathogenic bacteria in the dips, this was not significantly greater than that achieved in the control treatment. However, *B. cereus* has been controlled by all the probiotic bacteria to much lower level (from log 8 to around log 2) than that achieved in the control treatment (Table 5.7). This indicates that the lactic, acetic and citric acid present in the product acts as a preservative. It also appears that the probiotic bacteria also produce these acids as a metabolic by-product which plays a key role in the inhibition of pathogenic and spoilage bacteria. *B. cereus* was indicated to be very sensitive to acid level than the other pathogens tested in this

study (Table 5.1) and therefore, the additional acidity generated in the presence of probiotic bacteria may have contributed to the greater inhibition of *B. cereus* compared to the other pathogens.

5.3.3. Effect of probiotic bacteria on fungus

Many species of yeast have been identified as common spoilage organism of yoghurt and other fermented products (Pitt and Hocking, 1999; Loureiro and Kuerol, 1999). *Penecillium* spp., *Aspergillus* spp. and *Fusarium* spp. have been reported to produce toxins in food during storage (Filtenburg *et al.*, 1996; Samson *et al.*, 2000). These organisms are also observed to cause spoilage of dips. Therefore, *Penicillium* spp., *Aspergillus* spp. and *Fusarium* spp. were selected for this study. Many organic acids such as acetic, lactic, propionic, benzoic and sorbic acids are used in the food industry to control spoilage organism. However, benzoic and sorbic acids are the primary anti-fungal agents (Davidson, 2001). The commonly used anti-fungal agents such as natamycin (also known as pimaricin) are believed to be effective in controlling fungus. However, recent studies indicated that yeast and moulds develop some resistance to these agents (Brul and Coote, 1999; Lovreiro, 2000; Viljoen, 2001; Sanglard, 2002), suggesting that probiotic control of fungus may have advantages over the anti-fungal agents.

The anti-fungal effect of metabolites of probiotics (well diffusion assay) and active probiotic bacteria (spot and streak assay) on *A. niger*, *P. roqueforti*, *Fusarium* spp., *C. albicans* and *S. cerevisiae* are shown in Tables 5.8 and 5.9. There were differences in the degree of anti-fungal effects between the filter sterilized bacterial metabolites (well diffusion assay) and live bacteria (spot-on-streak assay). The anti-fungal effect of active probiotic bacteria when pre-grown for 48h anaerobically at 37°C before overlaying with agar inoculated with fungi was relatively stronger than that of filter sterilized metabolites. This suggests that continuous production and a large quantity of the anti-fungal metabolites of the bacteria is needed to inhibit the growth of yeast and mould. The filter-sterilized metabolites did not control any of the two yeast strains (Table 5.8).

The pre-grown active bacterial culture showed limited inhibition on *C. albicans* by *L. acidophilus* strain LAC1, *B. animalis* strain BB12, *L. paracasei* subsp. *paracasei* strain LC01, *L. casei* strain YLC and all strains of *L. rhamnosus* but did not show any

control over *S. cerevisiae* (Table 5.9). However, when co-cultured together in broth media with probiotic bacteria, *L. paracasei* subsp. *paracasei* LCS1, *L. rhamnosus* strains GG and LR 1524 inhibited *S. cerevisiae* (Figure 5.2). *C. albicans* was controlled by *B. animalis* BB12, *L. paracasei* subsp. *paracasei* strains LCO1, LCS1 and all strains of *L. rhamnosus* (LC705, LBA, LGG and LR 1524) while *L. acidophilus* showed only a limited control on *C. albicans*. This suggests that in a co-culture with the yeast *C. albicans*, continuous production of anti-microbial metabolites by these bacteria that closely contact with the yeast may have contributed to greater anti-mycotic effect. The above observation could be of clinical importance in treating candida infection caused by *C. albicans* in human subjects. Resent publications suggest that *L. acidophilus* could be used to treat candida infections but other strains tested such as *B. animalis* strain BB12, *L. paracasei* subsp. *paracasei* strain LC01 and LCS1, *L. rhamnosus* strains LC705, LBA, GG and LR 1524 showed far better control over *C. albicans*. The strains LCS1, LGG and LR produce comparatively larger quantities of lactic acid and moderate amount of acetic acid and phenyl lactic acid. The strain LR1524 produced considerably larger quantities of benzoic acid and phenyl lactic acid than the other strains (Table 5.3). The anti-fungal effects of acetic acid, benzoic acid and phynyllactic acid in controlling *S. cerevisiae* and the yeast fungi *C. albicans* may have been facilitated by lower pH level created by lactic acid and by the lipophilic nature of these acids. *P. freudenreichii* subsp. *shermanii* did not control *S. cerevisiae* but controlled *C. albicans* slightly. *S. cerevisiae* exhibited maximum level of resistance against the antagonistic effects of probiotic bacteria. These finding shows that *S. cerevisiae* could be included in a probiotic consortium success fully with the above mentioned probiotic bacteria in products where yeast is not considered as a spoilage organism.

In both assays, the inhibition of probiotic bacteria was strongest against *Fusarium* spp., moderate against *P. roqueforti* and very minimal against *A. niger*. Lavermicocca *et al.* (2002) reported that the minimal fungicidal concentration ($mg.mL^{-1}$) of phenyllactic acid for *Fusarium* spp., *P. roqueforti* and *A. niger* are 3.75, 5.00 and >10.00 respectively, indicating that different mould species exhibit different level of resistance against inhibitory metabolites. The slow growth of *Fusarium* spp. may also have contributed to the greater control by the bacteria and its metabolites. Out of the 5 probiotic bacterial

types, *L. casei*, *L. paracasei* subsp. *paracasei* and *L. rhamnosus* showed the greatest level of inhibition against all three mould species and on the yeast *C. albicans*. Although, the relatively faster growing mould, *A. niger* was the least controlled fungus by probiotics, actively growing cells of *P. freudenreichii* subsp. *shermanii* and *L. rhamnosus* strains GG and LR1524 showed moderate inhibition against *A. niger*. Out of all the bacteria tested, *L. rhamnosus* strain LR1524 produced larger quantities of acetic, lactic, butyric, benzoic and phenyl lactic acids. The ability to produce greater concentration of these acids may have contributed to greater inhibitory effect of *L. rhamnosus* strain LR1524 against spoilage and pathogenic bacteria yeast and moulds.

When the mould spores and yeast were grown with probiotics in RSM media for 18 h at 30°C and stored at 4°C for 2 days, there were no colonies of *P. roqueforti* and *Fusarium* spp. found in any of the fungi-bacterial co-cultures (complete inhibition by all bacteria). A moderate number of A. *niger* colonies (moderate inhibition) were found on 3M yeast and mould petrifilms (Table 5.10). After 15 days, *A. niger* has disappeared completely in all co-cultures except with *P. freudenreichii* subsp. *shermanii* strain PS1 while *P. roqueforti* started to reappear in all co-cultures (Table 5.10). Except *P. freudenreichii* subsp. *shermanii* strain PS1, all cultures have inhibited or caused lethal injury to *A. niger* on 3M yeast and mould petrifilms (even after 5 days). However, the level of inhibition on *P. roqueforti* was drastically reduced by day 15 resulting in an increased population for *P. roqueforti* (Table 5.10). These results suggest that when germination is controlled, *A. niger* does not grow at 4°C and loses its resistance for the metabolites of probiotics. However, *P. roqueforti* may have the ability to grow at 4°C. Therefore, the surviving spores of *P. roqueforti* might have started to re-grow slowly, resisting the presence of metabolites. This mould might either have developed some resistance to the inhibitory substance/s or the inhibitory substances might have been metabolised or disappeared. Nielson and Deboer (2000) found that *P. roqueforti* develops resistance against benzoic acid. The anti-fungal properties of phenyllactic and hydroxyphenyllactic acids were reported by Lavermicocca *et al.* (2000). Gummalla and Broadbent (2001) reported that *L. casei* produced phenyllactic acid, 4-hydroxy-phenyllactic acid and benzoic acid as a result of catabolism of tyrosine and phenylalanine. Gummalla and Broadbent (2001) also reported that phenyllactic acid

disappears in the supernatant of *L. casei* presumably used up in the production of phenyl acetic acid, benzoic acid and phenylalanine. Hillenga *et al.* (1995) and Hwang *et al.* (2001) indicated that *Penicillium chrysogenum* uses phenyl acetic acid (another strong anti-fungal agent as a precursor for penicillin G. The growth of mould after 15 days could have been results of a combination of reduction in the quantity of phenyllactic acid, the absence of phenyl acetic acid and the possible resistance developed by *P. roqueforti* to benzoic acid. The effects of bacterial cultures inoculated in dips on controlling yeast and mould area discussed next.

French onion dips were contaminated individually with strains of yeast (*C. albicans* at 10^4 cfu g^{-1}) and mould (*A. niger*, *P. roqueforti* and *Fusarium* spp. at 10^3 cfu g^{-1}), and were then inoculated with individual probiotic bacteria (in freeze dried or frozen forms). None of the bacteria showed noticeable control on yeast and mould. As indicated in Chapter 3, probiotics show some synergistic effect in survival, when grown in combination. ABCP or ABRP were two successful bacterial combinations identified. To ascertain whether the bacterial function is boosted by growing them in these combinations in dips, the experiment was repeated with the two bacterial combinations (ABCP; *L. acidophilus* strain LAC1, *L. paracasei* subsp. *paracasei* strain LCS1, *B. animalis* strain BB12 and *P. freadenreichii* subsp. *shermanii* strain P) and ABRP; *L. acidophilus* strain LAC1, *L. rhamnosus* strain LC 705, *B. animalis* strain BB12 and *P. fredenreichii* subsp. *shermanii* strain P) of probiotic bacteria (at 10^8 cfu g^{-1}) and stored at 4°C. A control treatment without probiotic bacteria was used to compare the results. There was no difference in the number of colony forming units (cfu g^{-1}) between the control and the treated dips over the first week. From week 3, A. *niger* colonies started to decline gradually. In week 5, both, the treated and the control dips with *P. roqueforti, Fusarium* spp and *C. albicans* did not produce any colonies in 3M yeast and mould petrifilms but the containers with control dip showed signs of swelling (data not shown). However, after 6 weeks of storage, the control dips started to show visible colonies of yeast and mould on the surface while the dips inoculated with probiotics did not show any visible colonies despite slight swelling of the container, indicating some biological activities of yeast and mould. However, when the 6-week old treated dips were plated on yeast and mould petrifilms, only a few *A. niger* colonies appeared but no other yeast and

mould grow. Although, this indicates that, in dip conditions the probiotic bacteria impose some control over the yeast and mould, it does not appear to be sufficient enough to prevent total biological activity of yeast and mould (swelling). Although, the probiotic bacteria were found to survive is large numbers in dips (Chapter 3), a lack of production/expression of inhibitory metabolites in sufficient quantity appears to weaken the ability to control yeast and mould. The prospects of having greater amount of bacterial metabolites in controlling yeast and mould is discussed in the next paragraph.

When the metabolites of individual probiotic bacteria were added at 5% (w/w) to dips, yeast and mould was controlled to varying degrees. In the control dip (without metabolites), the surface was very heavily covered with colonies of *P. roqueforti* and *C. albicans*. Metabolites of *L. acidophilus* strain LAC1, *B. animalis* strain BB12 and *P. fredenreichii* subsp. *shermanii* strain P did not produce any visible colonies on the surface throughout 10 weeks storage period (total control). *L. acidophilus* strain LA5 did not show any colonies of *P. roqueforti* on the surface until 8 weeks in storage but a few colonies started to appear on week 10. In the dips with metabolites of *L. paracasei* subsp. *paracasei* and *L. rhamnosus* strains *P. roqueforti* started to form visible colonies at the end of 6 weeks. However in the control dip (without metabolites) the dip surface was very heavily covered with colonies of *P. roqueforti* and *C. albicans* to successfully inhibit or hold back the growth of yeast and mould, an effective control is needed to prevent the establishment of fungal species at an early stage. Once the yeast or mould is established, it is difficult to control them even by using bacterial metabolites. These results indicate that a pre-grown bacterial culture that contains its metabolites appear to control yeast and mould, more effectively than inoculating bacteria cultures available in commercial forms (freeze-dried of frozen forms).

When the tubs of dips contaminated with yeast or moulds and inoculated with individual bacterial metabolites were removed from the fridge (4°C) after 1 week and kept at room temperature, colonies of *P. roqueforti* appeared on the surface of the dip in 3 days (Table 5.11). However, when these dips were removed from fridge after 4 weeks and then kept at room temperature, *P. roqueforti* colonies appeared in 2 days. This suggests that *P. roqueforti* would have started to grow early (during refrigeration) or have developed some resistance to the metabolites during storage. But, *A. niger*, *Fusarium* spp.

and *C. albicans* did not form any colonies on the surface even after 5 days at room temperature, either after refrigeration for 1 week or 4 weeks, indicating an effective and early control of them by the metabolites. Dips with metabolites of *L. acidophilus* strain LAC1 and *P. fredeunreichii* subsp. *shermanii* strain P did not form any colonies for 5 days at room temperature, either after 1 week or 4 of refrigeration. In contrast, the control dip and dips with metabolites of *L. paracasei* subsp. *paracasei* and *L. rhamnosus* showed formation of colonies after 2 days at room temperature, after 4 weeks of refrigeration. *P. roqueforti* was the first mould colony to appear (Table 5.11). These results confirms the earlier finding that of *L. acidophilus* strain LAC1 and *P. freudenreichii* subsp. *shermanii* strain P are very effective in inhibiting the growth of yeast and mould at an early stage and sustain the control at least for 10 weeks (not analysed for further period).

When treated with catalase and protiase enzymes, no effect on inhibition was observed with protease enzyme but the inhibitory effect was reduced with catalase enzyme only for strains of *L. acidophilus*. *L. acidophilus* produced relatively larger quantities of benzoic acid and phenyl lactic acid compared with *L. casei* group of bacteria (Table 5.7). Lavermicooa *et al.* (2003) reported that the anti-mycotic effect of phenyl lactic acid (pKa= 3.46) is mainly fungicidal and due to the lipophilic property of this acid. At low pH, phenyl lactic acid can diffuse into the fungal cells and cause death. Large amount of lactic acid and moderate quantities of acetic, benzoic and phenyl lactic acids produced by *L. acidophilus* strain LAC1 may have assisted in the control of moulds. *P. freudenreichii* subsp. *shermanii* strain P, produced little amount of lactic acid (Table 5.7) but large quantities of acetic, propionic, benzoic, formic and phenyl lactic acids, thus at low pH (4.3) of the dip lipophilic properties of formic, phenyl lactic and other acids (Table 5.7) and the acid effect of them may have involved in the control of yeast and moulds. *L. casei* and *L. paracasei* subsp. *paracasei* group of bacteria, might not have produced sufficient quantities of acetic, benzoic and phenyl lactic acid produced (Table 5.7) thereby they failed to inhibit the spore germination of yeast and mould entirely. Although, *L. rhamnosus* produced moderate quantity of phenyl lactic acid, the quantity of lactic acetic and benzoic acids produced by this bacterium might not have been enough to control the mould growth. Hence, the survived spores might have

produced colonies of yeast and mould when the inhibitory effect was further reduced at low storage temperature of 4°C.

The pronounced inhibitory effect of *L. rhamnosus* found against *C. albicans* and strains of moulds in the previous assays using agar plates, contradict with this finding. This enhanced inhibition in agar plates might have been due to the ability of *L. rhamnosus* to proliferate quickly within short period to produce inhibitory metabolites than that of the slow growing *L. acidophilus*. Further, *L. rhamnosus* might also have produced some other anti-microbial compounds that are effective against even *C. albicans* at higher pH and at higher temperatures. The agar plate experiments suggested that *L. rhamnosus* to be the best candidate as a controlling agent against yeast and mould. However, for acidic food products such as dips that are stored at refrigeration temperatures for longer storage periods, the bacterial metabolites or the stationary phase cultures of *L. acidophilus* strain LAC1 and *P. freudenreichii* subsp. *shermanii* strain P are the most suitable bio-preservatives, followed by that of *L. rhamnosus*. The inclusion of metabolites of *P. fredenreichii* subsp. *shermanii* strain P together with that of strains of *L. rhamnosus* might control the late re-growth of *P. roqueforti*. When the product's pH is low, benzoic and phenyl lactic acid in the metabolites of probiotic bacteria appeared to play major role in the inhibition of moulds. In a comparison, inoculation of late log phase or early stationary phase cultures of *L. acidophilus* strain LAC1 together with *P. freudenreichii* subsp. *shermanii* strain P produced similar effect of that achieved from two commercial bio-preservatives, Bioprofit (contains *L. rhamnosus* and *P. fredenreichii* subsp. *shermanii*) and ALC^{+} (contains *L. plantarum, Staphylococcus xylosus* and *Pediococcus* spp.) (data not shown).

5.4. Conclusion

Between and within spp./strain antagonism influenced the survival of probiotics in food products. Significant antagonistic effects occurred by commonly used probiotic *L. paracasei* spp. *paracasei* and *L. rhamnosus* towards other widely used probiotic *L. acidophilus*, *B. animalis* and *P. freudenreichii* subsp. *shermanii*. *L. rhamnosus*. Therefore, if *L. paracasei* spp. *paracasei* and/or *L. rhamnosus* is selected to be used in

combination with *L. acidophilus* and/ or *B. animalis* in a food product, care should be taken in deciding the ratio of these bacteria. It is suggested that to overcome the effect of antagonism in resulting sufficient number of bacteria in the food product, the later two need to be inoculated at least 1 log higher than that of the former two. The antagonism of *B. animalis* appeared to be dose dependent and therefore, the dosage should not be more than two log of others to have sufficient number of *L. acidophilus* and *L. rhamnosus* in the product. Of the strains tested, *L. paracasei* spp. *paracasei* was identified to be the most resistant to antagonism by other bacteria. Strain LAC1 performed best among *L. acidophilus* strains. Therefore, *L. acidophilus* strain LAC1, *B. animalis* Strain BB12 and *L. paracasei* spp. *paracasei* strains either LC01 or LBC81 is the best combination. All the probiotic bacteria tested possess varying degrees of inhibition towards spoilage and pathogenic bacteria. Spore formers and Gram positive bacteria were affected more than Gram negative bacteria. Organic acids such as lactic, formic, acetic, propionic, benzoic and phenyl lactic acids, produced by the bacteria appeared to play important role in inhibiting pathogenic bacteria. In dips, probiotic bacteria played a limited role in inhibiting pathogenic bacteria such as *E. coli*, *S. typhymurium* and *S. aureus*. However, they showed considerable inhibitory effect against *B. cereus* and *P. aeroginosa* in dips. Since, acetic, citric and lactic acids are components of dips, the natural dip pH is acidic (4.3-4.4). Therefore, inclusion of probiotic bacteria in frozen or freeze-dried form (commercially available forms) in the dip does not appear to add to the inherent inhibitory properties of dips. Since, the metabolites of probiotics controlled yeast and mould that grow well under acidic conditions, it is suggested that addition of metabolites of probiotics or late log phase or early stationary phase probiotic cultures containing their metabolites may also provide additional protection against pathogenic bacteria.

Probiotic bacteria *L. acidophilus*, *B. animalis*, *L. paracasei* spp. *paracasei*, *L. rhamnosus* and *P. freudenreichii* subsp. *shermanii* show remarkable antagonistic effect against yeast and mould. These bacteria affected moulds such as *A. niger*, *P. roqueforti* and *Fusarium* spp. more than yeast such as *S. cerevisiae* and *C. albicans*. Low pH, lipophilic properties of the organic acids and hydrogen peroxide (mainly by *L. acidophilus* and *B. animalis*) are suggested to caused the inhibitory effect against yeast and mould in food products.

Controlling fungi after it is established in food, by probiotic bacteria is difficult. Probiotic metabolites are needed in sufficient concentrations to kill or inhibit the germination of spores. Therefore, by reducing the initial contamination by practicing HACCP program to control the critical control points of the production is of paramount importance. In addition to this, by inoculating the food product with late log phase or early stationary phase cultures of selected probiotic bacteria will provide additional control of yeast and mould during storage, while maintaining sufficient number of probiotic bacteria in the product to produce health benefits to the consumer. It is concluded that *L. acidophilus* strain LAC1, *P. freudenreichii* subsp. *shermanii* strain P and *L. rhamnosus* added in the form of late log phase or early stationary phase cultures are suitable bio-preservatives for acidic food like French onion dip in providing good protection against spoilage and pathogenic organism while adding the benefits of probiotics to consumers.

Table 5.1 Minimal growth conditions for some psychrotrophic microorganisms (adapted from US Food and Drug Administration. Center for Food Safety and Applied Nutrition, Fish and Fisheries Products. Hazards and Controls Guidance, Third edition June 2001, Appendix 4)

Pathogenic and spoilage microorganisms	Lowest growth temperature °C	Minimum growth pH
Listeria monocytogenes	-0.4	4.4
Clostridium botulinum	3.3 (Produces toxin above 8°C)	5.0
Enterotoxigenic *Escherichia coli*	6.5	4.0
Bacillus cereus	5.0	4.3
Staphylococcus aureus	7.0 (Produces toxin above 10°C)	4.0
Salmonella spp.	5.2	3.7
Pseudomonas spp.	1.0	5.0
Yeast and mould	-4.0	≤2.0

Table 5.2 The diameter of zone of inhibition (mm) produced by antagonism among probiotic bacteria; *L. acidophilus, B. animalis, L. paracasei* subsp. *paracasei, L. rhamnosus* and *P. freudenreichii* subsp. *shermanii* using the spot-on-lawn assay

Producer organism/ Strain	Indicator organisms													
	L. acidophilus				*B. animalis*			*L. paracasei* subsp. *paracasei*			*L. rhamnosus*		*P. fredenreichii* subsp. *shermani*	
	LA5	LAC1	74-2	LAC4	BB12	BLC1	BF420	LC01	LCS1	LBC81	LC705	LBA	PS1	10360
L. acidophilus														
LA5	10	0	9	25	14	11	15	9	0	0	10	0	11	11
LAC1	15	10	20	20	24	22	22	20	18	15	0	12	0	0
74-2	12	9	9	12	14	11	20	9	0	0	10	0	0	0
LAC4	12	9	9	25	14	15	17	9	0	0	0	0	0	0
B. animalis														
BB12	0	0	0	0	0	0	10	9	0	0	15	0	9	9
BLC1	0	0	0	0	0	0	10	0	0	0	15	0	0	0
BF 420	0	0	0	0	0	0	11	0	0	0	15	0	0	0
L. paracasei subsp. *paracasei*														
LC01	16	11	20	13	22	22	26	15	11	15	20	12	15	18
LCS1	20	12	23	14	28	25	26	15	13	15	15	12	15	16
LBC81	15	11	18	16	23	22	23	17	12	17	15	12	12	12
L. rhamnosus														
LC705	17	12	20	14	23	25	28	16	13	17	18	13	15	30
LBA	18	12	18	15	26	20	31	17	13	15	15	13	20	30
P. freudenreichii subsp. *shermanii*														
PS1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
10360	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Table 5.3 Concentration of organic acids in μg g^{-1} and in mM (parenthesis) in overnight-grown culture supernatant of probiotic bacteria; *L. acidophilus*, *B. animalis*, *L. casei*, *L. paracasei* subsp. *paracasei*, *L. rhamnosus*, and *P. freudenreichii* subsp. *shermanii*

Probiotic bacteria/ Strain	pH	Organic acid μg g^{-1} and (mM)						
		Acetic acid pKa = 4.76	Formic acid pKa = 3.75	Lactic acid pKa = 3.86	Propionic acid pKa = 4.87	Benzoic acid pKa = 4.20	Butyric acid pKa = 4.82	Phenyllactic acid pKa = 3.46
L. acidophilus								
LA5	4.27	2820.2 (47.0)		7088.1 (78.8)		394.5 (3.26)		277.3 (1.68)
LAC1	3.90	2691.5 (44.9)		11383.5 (126.5)		246.1 (2.0)		335.3 (2.03)
B. animalis								
BB12	4.15	3024.9 (50.4)	18.7 (0.41)	2552.3 (30.9)		142.1 (1.17)	68.8 (0.78)	159.5 (0.97)
BLC1	3.82	3392.9 (56.6)	879.3 (19.1)	2862.6 (31.8)		95.8 (0.8)	79.4 (0.90)	
L. casei and *L. paracasei* subsp. *paracasei*								
LCO1	3.94	1614.9 (26.9)		7984.1 (88.7)		77.1 (0.64)	28.8 (0.33)	121.9 (0.74)
LCS1	3.87	1817.1 (30.3)		9356.4 (103.9)		57.7 (0.48)	17.7 (0.20)	181.3 (1.10)
YLC	3.73	1582.2 (26.4)		9546.8 (106.1)		87.0 (0.72)	38.0 (0.43)	177.4 (1.08)
L. rhamnosus								
LC705	4.01	1582.9 (26.4)		6935.1 (77.1)		41.09 (0.34)	41.3 (0.47)	207.9 (1.26)
LBA	3.74	1586.3 (26.4)		9388.7 (104.3)		101.3 (0.84)	42.7 (0.49)	302.3 (1.83
LGG	3.86	1670.7 (27.9	72.3 (1.6)	8859.7 (98.4)		33.3 (0.28)	14.9 (0.17)	290.4 (1.76)
LR1524	3.92	1763.9 (29.4)		9203.7 (102.3)		209.6 (1.73)	67.2 (0.76)	683.4 (4.14)
P. freudenreichii subsp. *shermanii*								
P	5.97	2435.9 (40.6)	1336.2 (29.1)	2156.4 (23.9)	1902.9 (25.7)	281.5 (2.33)	109.9 (1.25)	128.3 (0.78)
PS1	6.27	1892.9 (31.55)	947.7 (20.60	2965.8 (32.9)	1800.3 (24.3)	272.8 (2.25)	97.5 (1.11)	73.0 (0.44)
PB10360	6.20	2298.7 (38.3)	863.5 (18.8)	1118.9 (12.4)	2600.0 (35.1)	218.9 (1.81)	78.6 (0.89)	32.9 (0.2)

Table 5.4 Diameter of zone of inhibition (mm) produced by probiotic bacteria; *L. acidophilus*, *B. animalis*, *L. casei*, *L. paracasei* subsp. *paracasei*, *L. rhamnosus* and *P. freudenreichii* subsp. *shermanii* on pathogenic and spoilage bacteria; *E. coli*, *S. typhimurium*, *S. aureus*, *P. aeroginosa*, *B. cereus* and *B. stearothermophilus* using spot on lawn assay

Producer organism	Anaerobic incubation at 37° C for 72 h						Anaerobic incubation at 37° C for 72 h followed by aerobic incubation at 37° C 24 h	
	E. coli	*S. typhymurium*	*S. aureus*	*B. cereus*	*B. stearothermophilus*[#]	*P. aeruginosa*[#]	*B. stearothermophilus*	*P. aeruginosa*
L. acidophilus								
LA5	13	14	15	15	NG	NG	19	25
LAC1	17	14	18	20	NG	NG	33	27
B. animalis								
BB12	15	14	14	16	NG	NG	24	21
BLC1	14	12	12	14	NG	NG	15	21
L. casei and *L. paracasei* subsp. *paracasei*								
LC01	13	14	14	16	NG	NG	30	14
LCS1	15	12	15	17	NG	NG	28	29
YLC	14	13	14	16	NG	NG	33	25
L. rhamnosus								
LC705	11	13	13	17	NG	NG	36	29
LBA	12	12	15	20	NG	NG	33	26
LGG	15	14	23	17	NG	NG	34	25
LR1524	15	15	21	19	NG	NG	38	35
P. fredenreichii subsp. *shermanii*								
P	0	0	12	0	NG	NG	15	0
PS1	0	0	15	0	NG	NG	12	0
PB 10360	0	0	12	0	NG	NG	0	0

[#] Since *B. stearothermophilus* and *P. aeruginosa* did not grow in the 72-h aerobic phase of the experiment, they were further tested under 24-h aerobic conditions. NG= no growth

Table 5.5 Effect of co-culturing with probiotic bacteria in reconstituted skim milk media on log population of pathogenic and spoilage bacteria

Probiotic bacteria/Strain	Pathogenic bacteria			
	E. coli	*S. typhimurium*	*S. aureus*	*B. cereus*
L. acidophilus				
LA5	7.0	7.5	6.6	5.6
B. animalis				
BB12	7.0	4.7	4.7	4.0
L. paracasei subsp. *paracasei*				
LC01	7.0	5.9	5.3	4.0
LCS1	7.0	5.7	5.8	4.0
L. rhamnosus				
LC705	7.6	4.0	5.0	4.6
LBA	7.0	4.0	5.3	4.3
P. freudenreichii subsp. *shermanii*				
P	7.6	7.3	6.6	4.7
PS1	8.0	7.7	8.6	5.7
Control (no probiotic)	8.9	9.1	8.6	8.2

Table 5.6 Diameter of zone of inhibition (mm) produced by probiotic bacteria; *L. acidophilus*, *B. animalis*, *L. casei*, *L. paracasei* subsp. *paracasei* and *L. rhamnosus* on pathogenic and spoilage bacteria; *E. coli*, *S. typhimurium*, *P. aeruginosa*, *S. aureus*, *B. cereus* and *B. stearothermophilus* in well diffusion assay

Producer organism / Strain	Indicator organism					
	E. coli	*S. typhimurium*	*P. aeruginosa*	*S. aureus*	*B. cereus*	*B. stearothermophilus*
L. acidophilus						
LA5	15 (23)[#]	13 (23)	10 (25)	14 (23)	12	10 (18)
LAC1	11 (23)	10 (20)	13 (27)	12 (21)	28	12 (20)
B. animalis						
BB12	11 (24)	13	13 (21)	15 (24)	11	15 (21)
BLC1	15 (25)	11 (23)	10 (20)	0	10	12 (22)
L. casei and *L. paracasei* subsp. *paracasei*						
LCO1	12 (21)	14 (24)	15 (25)	10 (23)	15	15
LCS1	11 (23)	13 (22)	14 (29)	17 (25)	13	14 (24)
YLC	15 (24)	15 (27)	15 (25)	13 (27)	15	11 (27)
L. rhamnosus						
LC705	9	12	26	12	18	0
LBA	14	0	27	11	18	11
LGG	14 (24)	14 (25)	16 (34)	15 (24)	15	15 (28)

[#] Values in parenthesis denote zones of inhibition plus zone of diffusion

Table 5.7 Effect of co-culturing with probiotic bacteria in French onion dip on log population of pathogenic and spoilage bacteria inoculated at a rate of log 8 cfu g^{-1}

Probiotic bacteria/Strain	Pathogenic bacteria			
	E. coli	*S. typhimurium*	*S. aureus*	*B. cereus*
L. acidophilus				
LA5	4.6	4.5	4.5	2.3
B. animalis				
BB12	4.7	4.6	4.7	<2
L. casei and *L. paracasei* subsp. *paracasei*				
LC01	4.8	4.8	4.7	2.5
LCS1	4.8	4.8	4.8	<2
YLC	4.8	4.7	4.8	<2
L. rhamnosus				
LC705	4.9	4.8	4.7	<2
LBA	4.7	4.7	4.5	2.0
LGG	4.7	4.6	4.5	<2
LR1524	4.8	4.7	4.8	2.9
Control (no probiotic)	5.0	5.0	5.0	4.0

Table 5.8 The grades of anti-fungal activity of *L. acidophilus*, *B. animalis*, *L. casei*, *L. paracasei* subsp. *paracasei*, *L. rhamnosus* and *P. fredenreichii* subsp. *shermanii* on fungal strains; *A. niger*, *P. roqueforti* and *Fusarium* spp., *C. albicans* and *S. cerevisiae* in well diffusion assay

Probiotic bacteria	Fungal strain				
	A. niger	*P. roqueforti*	*Fusarium* spp.	*C. albicans*	*S. scerevisiae*
L. acidophilus					
LA5	+	++	++	-	-
LAC1	+	++	++	-	-
B. animalis					
BB12	+	+	++	-	-
BLC1	-	-	++	-	-
L. casei and *L. paracasei* subsp. *paracasei*					
LC01	+	++	+++	-	-
LCS1	++	++	+++	-	-
YLC	++	++	+++	-	-
L. rhamnosus					
LC705	++	++	+++	-	-
LBA	++	++	+++	-	-
LGG	++	++	+++	-	-
LR1524	++	++	+++	-	-
P. freudenreichii subsp. *shermanii*					
P	+	+	++	-	-
PS1	+	+	++	-	-
Pb 1036	+	+	+	-	-
control	-	-	-	-	-

The anti-fungal were graded as follows: (-, no suppression; +, weak suppression on the wells; ++, detectable suppression around the wells; +++ with detectable clear zone around the well)

Table 5.9 The grades of anti-fungal activity of *L. acidophilus*, *B. animalis*, *L. casei*, *L. paracasei* subsp. *paracasei*, *L. rhamnosus*, and *P. freudenreichii* subsp. *shermanii* on fungal strains; *A. niger*, *P. roqueforti*, *Fusarium* spp. *C. albicans* and *S. cerevisiae*, in a spot and streak test (dual culture overlay system).

Probiotic bacteria/ Strain	Fungal strain				
	A. niger	*P. roqueforti*	*Fusarium* spp.	*C. albicans*	*S. cerevisiae*
L. acidophilus					
LA5	+	++	++++	-	-
LAC1	++	+++	++++	+	-
B. animalis					
BB12	+	++	++++	+	-
BLC1	+	+	++++	-	-
L. casei and *L. paracasei* subsp. *paracasei*					
LC01	++	++++	++++	+	-
LCS1	++	++++	++++	-	-
YLC	++	++++	++++	+	-
L. rhamnosus					
LC705	++	++++	++++	+	-
LBA	++	++++	++++	+	-
LGG	++	++++	++++	+	-
LR1524	+++	++++	++++	+	-
P. freudenreichi subsp. *shermanii*					
P	+++	++	+++	+	-
PS1	+++	++	+++	+	-
Pb 1036	+++	++	+++	-	-
control	-	-	-	-	-

The area of zones were graded as follow: -, no suppression, +, no fungal growth for 5 mm around the spot and streak, ++, no fungal growth for 10mm around the spot and streak, +++, no fungal growth for 15 mm around the spot and streak, ++++, no fungal growth for above 15 mm around the spot and streak

Table 5.10 Log_{10} population (cfu g^{-1}) of yeast and mold strains; *C. albicans*, *A. niger*, *P. roqueforti* and *Fusarium* spp. when probiotic bacteria and mould spore/ conidia were grown together in reconstituted skim milk for 18h at 30°C and stored at 4°C for 15 days

Probiotic bacteria	Days of storage/ Yeast and mould strains							
	After 1 day at 4°C				After 15 days at 4°C			
	A. niger	*P. roqueforti*	*Fusarium* spp.	*C. albicans*	*A. niger*	*P. roqueforti*	*Fusarium* spp.	*C. albicans*
L. acidophilus								
LA5	2.7	<1	<1	6.9	<1	2.5	<1	6.4
LAC1	2.1	<1	<1	6.9	<1	1.7	<1	5.8
B. animalis								
BB12	2.9	<1	<1	6.6	<1	<1	<1	5.9
BLC1	3.1	<1	<1	7.0	<1	3.5	<1	6.3
L. casei and *L. paracasei* subsp. *paracasei*								
LC01	1.5	<1	<1	6.6	<1	2.1	<1	5.0
LCS1	<1	<1	<1	6.6	<1	1.2	<1	5.1
YLC	<1	<1	<1	6.5	<1	2.1	<1	5.2
L. rhamnosus								
LC705	<1	<1	<1	6.1	<1	1.8	<1	3.6
LBA	<1	<1	<1	6.4	<1	<1	<1	5.1
LGG	1.6	<1	<1	6.1	<1	2.0	<1	5.0
LR1524	<1	<1	<1	5.9	<1	1.9	<1	4.3
P. freudenreichii subsp. *shermanii*								
P	3.6	<1	<1	6.2	<1	<1	<1	5.4
PS1	3.3	<1	<1	6.2	2.3	1.7	<1	6.8
Pb 10360	4.1	<1	<1	6.2	<1	2.0	<1	6.3
Control	4.3	4.0	4.0	8.9	2.9	3.7	<1	6.2

Table 5.11 Colony forming units (cfu g^{-1}) of mold strains; *A. niger* and *P. roqueforti* on the surface of French onion dip (in 100g container) inoculated with metabolites of probiotic bacteria (10% w/w), stored at 4 °C for 4 weeks and kept at room temperature for 2 or 3 days

Source of metabolite	Mould strain/ Days of exposure to air			
	A. niger		*P. roqueforti*	
Bacteria/Strain	2 days	3 days	2 days	3 days
L. acidophilus				
LA5	1	70	40	134
LAC1	0	0	0	0
L. paracasei subsp. *paracasei*				
LC01	1	70	1	18
LCS1	0	0	0	0
L. rhamnosus				
LC705	3	70	8	40
LBA	5	70	10	50
P. freudenreichi subsp. *shermanii*				
P	1	15	0	5
Control (no probiotic)	10	100	200	234

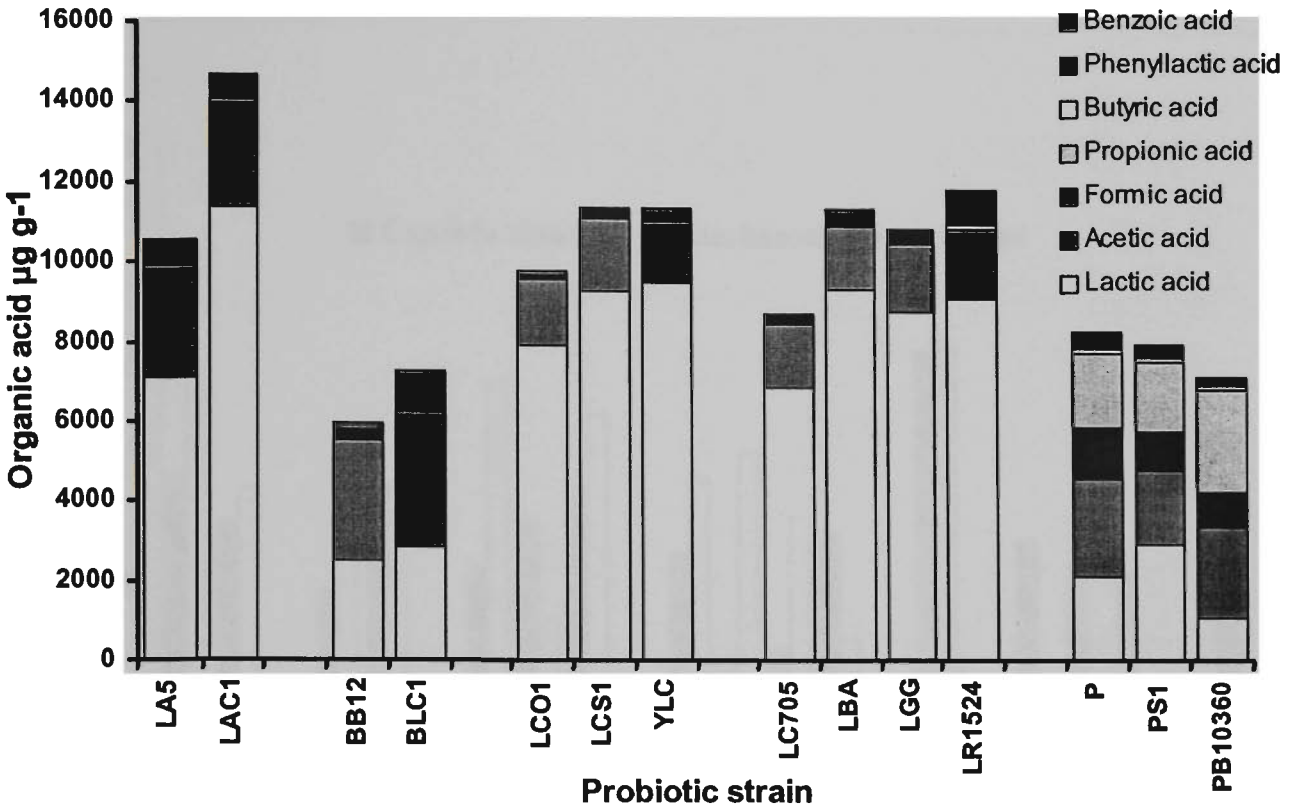

Figure 5.1 Proportion of organic acids (µg g^{-1}) in the overnight-culture supernatant of 2 strains of *L. acidophilus*, 2 strains of *B. animalis*, 1 strain of *L. casei*, 2 strains of *L. paracasei* subsp. *paracasei*, 4 strains of *L. rhamnosus* and 3 strains of *P. freudenreichii* subsp. *shermanii*

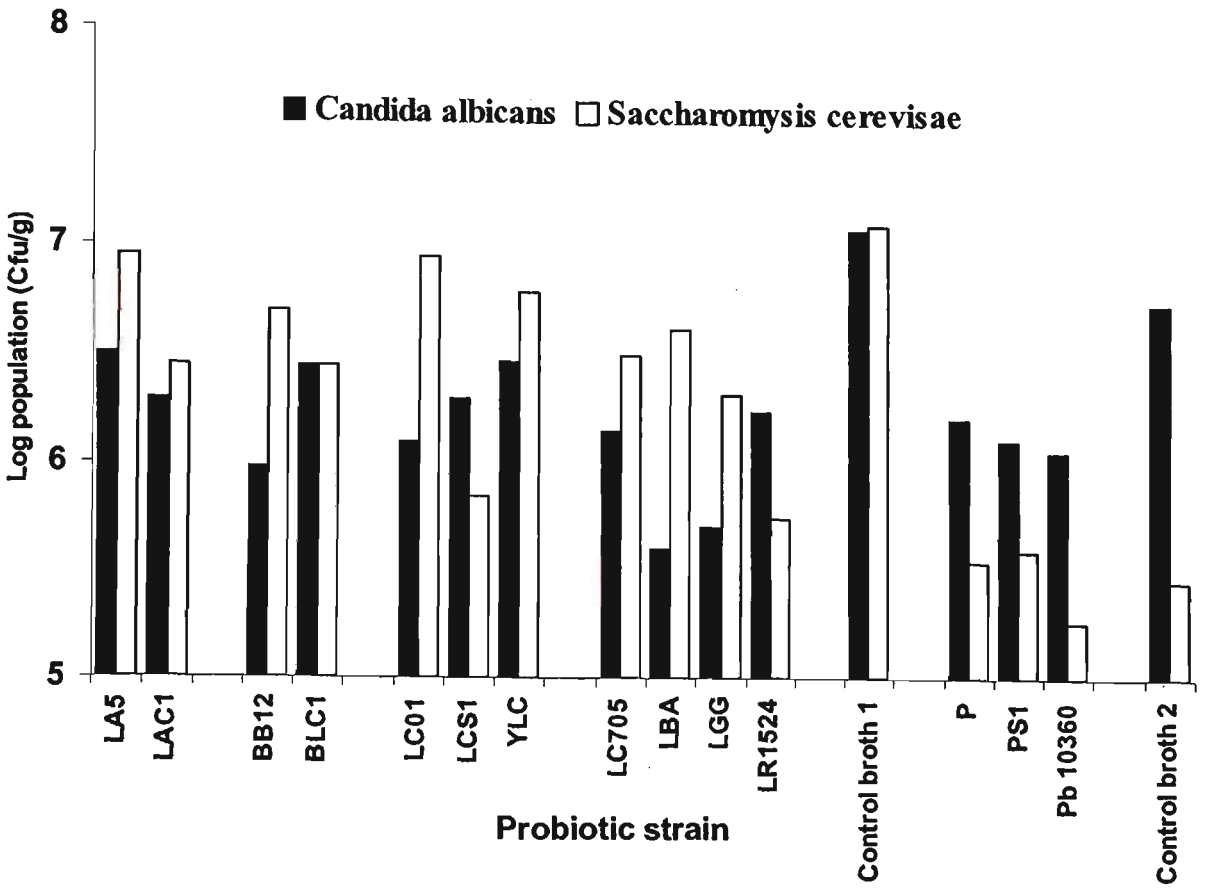

Figure 5.2 Effect of probiotic strains of 2 strains of *L. acidophilus,* 2 strains of *B. animalis*, 1 strain of *L. casei*, 2 strains of *L. paracasei* subsp. *paracasei*, 4 strains of *L. rhamnosus* and 3 strains of *P. freudenreichii* subsp. *shermanii* on log population of yeast strains *C. albicans* and *S. cerevisiae* co-cultured in broth culture

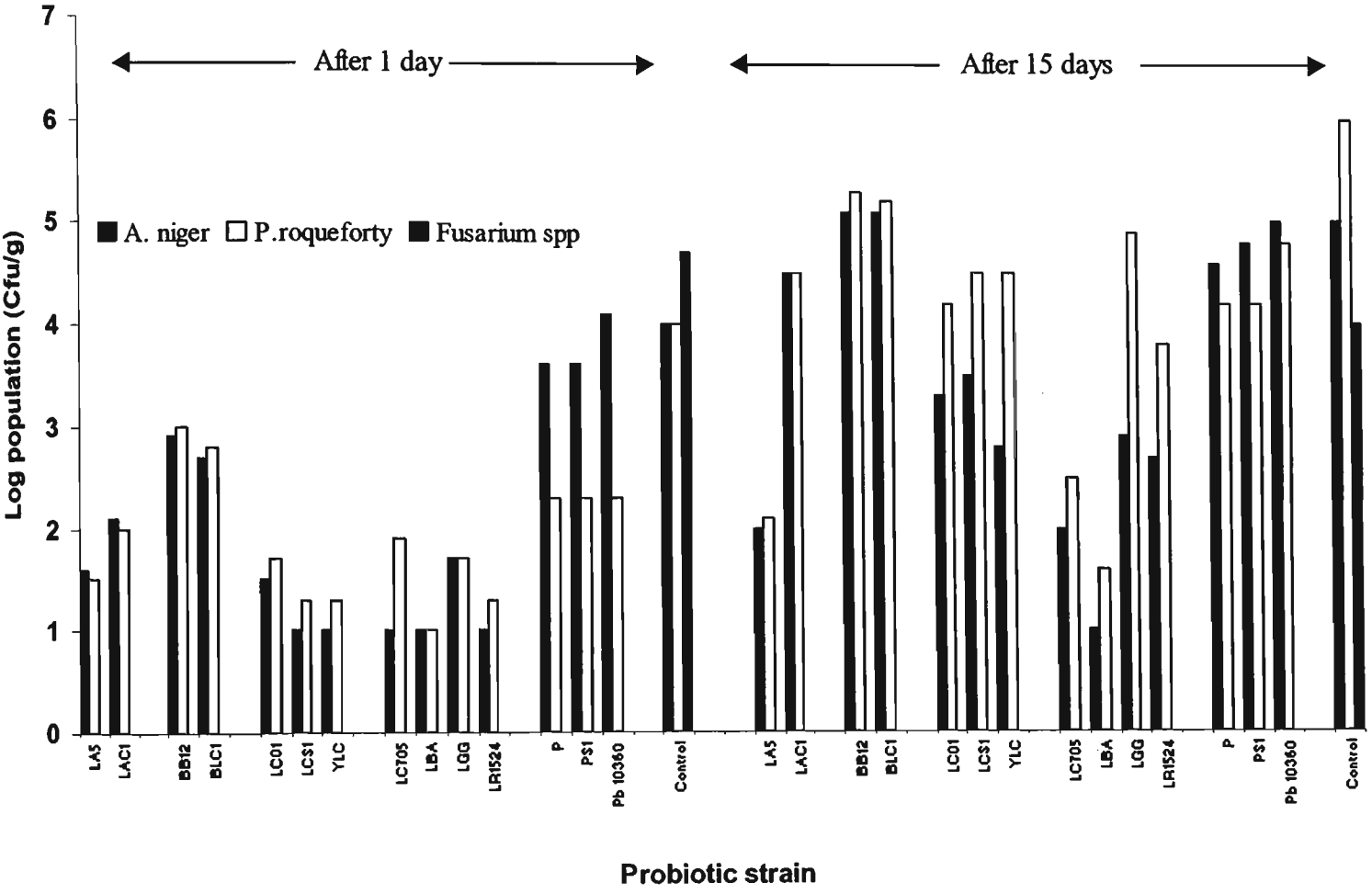

Figure 5.3 Log_{10} population (cfu g^{-1}) of mold strains; *A. niger*, *P. roquefori* and *Fusarium* spp. when probiotic bacteria and mould spore/ conidia were grown together in reconstituted skim milk for 18h at 30°C and stored at 4°C for 1 day and 15 days

CHAPTER 6

OVERALL CONCLUSIONS

Increasing the number of probiotic food products available to consumers is an effective means of increasing the chances of consuming probiotics by consumers in beneficial numbers (at least 10^7cfu.ml^{-1}). Exploring the potential of using cheese-based dips as carriers of probiotics is the main purpose of this study. As a standard practice in the preparation of dips, a mixture of acetic acid, lactic acid and citric acids are used to bring the pH of the dips to recommended level of 4.40-4.45. Oil and gums are also important ingredients of dips to improve their texture and flavour. Understanding the implications of these practices on the survival of probiotics in dips and in delivering them in sufficient numbers at the time of consumption is one of the major objectives of the study. The other major objective was to elucidate the factors associated with the ability of the probiotic bacteria in enhancing the inhibition of pathogenic spoilage organism in dips. In this regard, the study clearly indicated that cheese-based dips passed the double hurdle in qualifying themselves as a suitable carrier of probiotics, in that, it could maintain the probiotic population in the product for at least 10 weeks above the recommended level and that the probiotics acted as a biopreservative against spoilage organisms in the product.

The currently available methods used to enumerate bacteria have limitations in applying to accurately enumerate the numbers of individual bacteria in a consortium of probiotics. Primarily, the study has developed effective selective enumeration methods for specific probiotic cultures to enumerate their numbers and ensure the presence of probiotic organism. In addition to this, methods have also been developed to evaluate the ideal conditions in which the organisms survive better and elucidate the mechanism by which the probiotic organisms antagonise pathogenic and spoilage organisms. Some of these methodologies that can be used in similar studies are highlighted here.

The cultures of *L. delbrueckii* subsp. *bulgaricus*, *S. thermophilus*, *L. casei*, *L. rhamnosus*, *L. acidophilus*, *Bifidobacterium* spp. and propionibacteria were tested in selected bacteriological media to evaluate their suitability as selective media. Nineteen bacteriological media were tested at different incubation conditions in this study. Aerobic and anaerobic incubations were carried out at temperatures of 27° C, 30° C, 37° C, 43° C and 45° C for durations of 24-h, 72-h and 7-9 days. ST agar

aerobic incubation at 37° C for 24-h was identified as suitable for *S. thermophilus*. *L. delbrueckii* subsp. *bulgaricus* can be enumerated in MRS agar (pH 4.58 or pH 5.20) anaerobic incubation at 45° C for 72 h. MRS- V agar anaerobic incubation at 43° C for 72 h was suitable to enumerate *L. rhamnosus*. Anaerobic incubation in MRS-V agar at 37° C for 72 h was selective to enumerate *L. casei*. It is recommended that subtraction method should be implemented when *L rhamnosus* is present in the product. To do this, the count of *L. rhamnosus* recorded on MRS-V agar at 43° C for 72h under anaerobic incubation should be subtracted from the total count of *L. casei*. and *L. rhamnosus*, recorded on MRS-V agar 37° C for 72h under anaerobic incubation. *L. acidophilus* can be enumerated on MRS-agar at 43° C for 72h under anaerobic incubation or in MRS-maltose at 43° C under anaerobic incubation for 72h or on MRS-sorbitol agar 37° C for 72 h under anaerobic incubation. Bifidobacteria can be enumerated on MRS-NNLP (nalidixic acid, neomycine sulfate, lithium chloride and paramomycine sulfate) agar. Propionibacteria can be enumerated on sodium lactate (NaLa) agar. The subtraction method was the most suitable method for counting propionibacteria, in the presence of lactic acid bacteria in a product. In this method, day 3 count on NaLa agar under anaerobic incubation at 30°C of lactic acid bacteria, was subtracted from the day 7 count (total count) of lactic acid bacteria and propionibacteria in the same incubation conditions, to give the propionibacteria count. Errors can happen depending on the number and kinds of cultures present in the product. Thus, selecting the most suitable method for selective enumeration depending on the cultures present in the product is very important for accurate results.

The commonly used and recognised probiotic bacteria, *L. acidophilus, L. paracasei* subsp. *paracasei, L. rhamnosus, B. animalis,* and *P. freudenreichii* subsp. *shermanii* were evaluated for survival in dips for a maximum storage period of 10 weeks. Between- and within- species/strain antagonism is a major cause of loss of probiotics in a culture and is a major determinant of suitable bacterial combination. When used in combination, *L. acidophilus, B. animalis, L. paracasei* subsp. *paracasei,* and *L. rhamnosus* showed varied levels of antagonism, while *P. freudenreichii* subsp. *shermanii* showed no antagonistic effects. When grown individually, all these probiotic bacteria appear to grow in cheese-based French onion dip. However, the inoculation level should be at least 8 log for *L. acidophilus*

and *B. animalis* and 7 log for *L. paracasei* subsp. *paracasei*, and/ or *L. rhamnosus* to obtain greater than 6 log of individual bacterial population at the end of shelf life.

In terms of overall bacterial population, *L. acidophilus, L. paracasei* subsp. *paracasei*, *B. animalis,* and *P. freudenreichii* subsp. *shermanii* (ABCP) is the most suitable co-habitant probiotic consortium to be effectively used in dips. The combination of *L. acidophilus, L. rhamnosus*, *B. animalis,* and *P. freudenreichii* subsp. *shermanii* (ABRP) is the second best bacterial consortium to be used in dips. When inoculated at 9 $\log.g^{-1}$ or more, *L. acidophilus* and *B. animalis* population can be maintained above required level for health benefit over the storage period of 10 weeks. *L. paracasei* subsp. *paracasei*, *L. rhamnosus* and *P. freudenreichii* subsp. *shermanii* were not adversely affected by any of the bacteria in any combinations and can be inoculated at a rate of 7 log to maintain a population above 6 logs over the storage period of 10 weeks.

Of the strains tested, *L. paracasei* subsp. *paracasei* has been identified to be the most resistant to antagonism by other bacteria. Strain LAC1 performed best of *L. acidophilus*. Therefore, *L. acidophilus* strain LAC1, *B. animalis* strain BB12 and *L. paracasei* subsp. *paracasei* strains either LC01 or LBC81 is the best combinations. The common components of the dips, oils and gums, did not affect the survival of probiotics in cheese-based dips. The texture and flavour enhancing ingredients such as oils and gums can be used to make dips without affecting the survival of the probiotics.

When tested in co-cultures, all the probiotic bacteria were found to possess varying degrees of inhibition towards spoilage and pathogenic bacteria. Spore formers and Gram-positive bacteria are affected more than Gram negative bacteria. Organic acids such as lactic, formic, acetic, propionic, benzoic and phenyl lactic acids, produced by the bacteria appeared to play an important role in inhibiting pathogenic and spoilage bacteria. Although, probiotic bacteria survived in dips, due to limited growth and proliferation of these bacteria during cold storage these bacteria showed only limited inhibition against pathogenic bacteria such as *E. coli*, *S. typhiymurium* and *S. aureus*. However, these bacteria showed considerable inhibitory effect against *B. cereus* and *P. aeruginosa* in dips. Since, acetic, citric and lactic acids are components of dips, the natural dip pH is acidic (4.40-4.45). It is concluded that, inclusion of live probiotic bacteria in frozen or freeze-dried forms

(commercially available forms) did not enhance the inherent inhibitory properties of dips against pathogenic and spoilage bacteria.

Probiotic bacteria *L. acidophilus*, *B. animalis*, *L. paracasei* subsp. *paracasei*, *L. rhamnosus* and *P. freudenreichii* subsp. *shermanii* show remarkable antagonistic effect against yeast and mould. These bacteria affected the moulds such as *A. niger*, *P. roqueforti* and *Fusarium* spp. more than yeast such as *S. cerevisiae* and *C. albicans*. Low pH, lipophilic properties of the organic acids and hydrogen peroxide (mainly by *L. acidophilus* and *B. animalis*) inhibited yeast and mould in food products. In this study, the metabolites of probiotics controlled yeast and mould that grow well under acidic conditions. This suggests that additional protection against pathogenic bacteria can be achieved by the addition of metabolites of probiotics or late log phase or early stationary phase probiotic cultures containing their metabolites to dips.

Controlling the spread of fungi after it is established in food, by probiotic bacteria is difficult. Faster growth and proliferation of probiotic bacteria and accumulation of large quantities of probiotic metabolites are needed to kill or inhibit the germination of spores. Therefore, it is recommended that controlling the initial contamination of food products below the 'critical control points' by practicing appropriate HACCP program is paramount. In addition to this, by inoculating the food product with late log phase or early stationary phase cultures of selected probiotic bacteria can provide additional and long lasting control of yeast and mould during storage, while maintaining sufficient number of probiotic bacteria in the product to produce health benefits to the consumer. It is concluded that *L. acidophilus* strain LAC1, *P. freudenreichii* subsp. *shermanii* strain P and *L. rhamnosus* added in the form of late log phase or early stationary phase cultures are suitable bio-preservatives for acidic food like French onion dip in providing good protection against spoilage and pathogenic organism while adding the benefits of probiotics to consumers.

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APPENDICES

Figure 1 Inhibition zone produced by probiotic bacterial strains LR1524 (top left), LA5 (top right), LCS 1 (botton left and BB12 (bottom right) on *P. roqueforti* in a Spot and streak plate

J. Dairy Sci. 86:2288–2296

Selective Enumeration of *Lactobacillus delbrueckii* ssp. *bulgaricus*, *Streptococcus thermophilus*, *Lactobacillus acidophilus*, Bifidobacteria, *Lactobacillus casei*, *Lactobacillus rhamnosus*, and Propionibacteria

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ABSTRACT

Nineteen bacteriological media were evaluated to assess their suitability to selectively enumerate *Lactobacillus delbrueckii* ssp. *bulgaricus*, *Streptococcus thermophilus*, *Lactobacillus casei*, *Lactobacillus rhamnosus*, *Lactobacillus acidophilus*, bifidobacteria, and propionibacteria. Bacteriological media evaluated included *Streptococcus thermophilus* agar, pH modified MRS agar, MRS-vancomycine agar, MRS-bile agar, MRS-NaCl agar, MRS-lithium chloride agar, MRS-NNLP (nalidixic acid, neomycin sulfate, lithium chloride and paramomycine sulfate) agar, reinforced clostridial agar, sugar-based (such as maltose, galactose, sorbitol, manitol, esculin) media, sodium lactate agar, arabinose agar, raffinose agar, xylose agar, and *L. casei* agar. Incubations were carried out under aerobic and anaerobic conditions at 27, 30, 37, 43, and 45°C for 24, 72 h, and 7 to 9 d. *S. thermophilus* agar and aerobic incubation at 37°C for 24 h were suitable for *S. thermophilus*. *L. delbrueckii* ssp. *bulgaricus* could be enumerated using MRS agar (pH 4.58 or pH 5.20) and under anaerobic incubation at 45°C for 72 h. MRS-vancomycine agar and anaerobic incubation at 43°C for 72 h were suitable to enumerate *L. rhamnosus*. MRS-vancomycine agar and anaerobic incubation at 37°C for 72 h were selective for *L. casei*. To estimate the counts of *L. casei* by subtraction method, counts of *L. rhamnosus* on MRS-vancomycine agar at 43°C for 72 h under anaerobic incubation could be subtracted from total counts of *L. casei* and *L. rhamnosus* enumerated on MRS-vancomycine agar at 37°C for 72 h under anaerobic incubation. *L. acidophilus* could be enumerated using MRS-agar at 43°C for 72 h or Basal agar-maltose agar at 43°C for 72 h or BA-sorbitol agar at 37°C for 72 h, under anaerobic incubation. Bifidobacteria could be enumerated on MRS-NNLP agar under anaerobic incubation at 37°C for 72 h. Propionibacteria could be enumerated on sodium lactate agar under anaerobic incubation at 30°C for 7 to 9 d. A subtraction method was most suitable for counting propionibacteria in the presence of other lactic acid bacteria from a product. For this method, counts of lactic bacteria at d 3 on sodium lactate agar under anaerobic incubation at 30°C were subtracted from counts at d 7 of lactic bacteria and propionibacteria.

(**Key words:** *Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus rhamnosus*, propionibacteria).

Abbreviation key: BA = basal agar, **NNLP** = nalidixic acid, neomycine sulfate, lithium chloride and paromomycine sulfate, **ST agar** = *Streptococcus thermophilus* agar, **RCA** = reinforced clostridial agar, **RSM** = reconstituted skim milk.

INTRODUCTION

A number of health benefits have been claimed for probiotic bacteria and more than 90 probiotic products containing one or more groups of probiotic organisms are available worldwide. Probiotic food can be defined as "food containing live microorganisms which actively enhance the health of consumers by improving the balance of microflora in the gut" (Fuller, 1992).

A number of probiotic organisms including *L. acidophilus*, *Bifidobacterium* spp., *Lactobacillus casei*, *Lactobacillus rhamnosus*, and *Propionibacterium* are incorporated in dairy foods. These organisms grow slowly in milk during product manufacture. Therefore the usual practice is to incorporate yogurt bacteria (*Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*) along with probiotic cultures. Yogurt bacteria do not survive in the gastric passage or colonize in the gut (Shah and Jelen, 1990) and are unlikely to provide any therapeutic benefits. However, yogurt bacteria grow rapidly and thus are added to speed up the fermentation process.

To provide health benefits, the suggested concentration for probiotic bacteria is 10^6 cfu/g of a product (Shah, 2000). It seems reasonable to assume that the beneficial

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effects of probiotic bacteria can be expected only when viable cells are ingested. An important parameter in monitoring viable organisms in assessing product quality is the ability to count probiotic bacteria differentially. Differential enumeration of probiotic bacteria is difficult owing to the presence of several types of similar microbes in a product. In order to assess viability and survival of probiotic bacteria, it is important to have a working method for selective enumeration of these bacteria.

Several media for selective enumeration of *L. acidophilus* and *Bifidobacterium* spp. have been previously proposed (Hunger, 1986; Hull and Roberts, 1984; Laroia and Martin, 1991; Dave and Shah, 1996; Lankaputhra and Shah, 1996; Wijsman et al., 1989; Shah, 1997, 2000). Similarly, several media have been proposed for selective enumeration of yogurt cultures (Onggo and Fleet, 1993; Samona and Robinson, 1984). There are only few reports that have described selective enumeration of *Lactobacillus casei* in the presence of other probiotic bacteria and yogurt bacteria (Champagne et al., 1997; Ravula and Shah, 1998). Selective enumeration of *L. casei* from probiotic products based on a 15°C incubation temperature and 14 day incubation was studied by Champagne et al. (1997). Ravula and Shah (1998) developed a medium, known as LC agar, for selective enumeration of *L. casei*. Selective enumeration of *Lactobacillus reuteri*, *Lactobacillus plantarum*, *Lactobacillus rhamnosus*, and propionibacteria has not been studied extensively. The aim of this study was to develop and evaluate media for selective enumeration of *S. thermophilus*, *L. delbrueckii* ssp. *bulgaricus*, *L. acidophilus*, *L. casei*, *L. rhamnosus*, bifidobacteria, and propionibacteria.

MATERIALS AND METHODS

Bacteria Cultures and Propagation

L. delbrueckii ssp. *bulgaricus* (LB 100B), *S. thermophilus* (ST 2362), *L. casei* (DS 930), *L. acidophilus* (DS 910), *Bifidobacterium lactis* (DS920), and *Propionibacterium freudenreichii* ssp. *globosum* (type standard 10360) were provided by DSM Gist brocades (DSM Gist brocade Australia Pty. Ltd., Werribee, Australia). *L. paracasei* ssp. *paracasei* (LC01), *L. acidophilus* (LA 5), *Bifidobacterium lactis* (Bb 12), and *Propionibacterium freudenreichii* ssp. *shermanii* (PS1) were obtained from Chr. Hansen (Chr. Hansen Pty. Ltd., Bayswater, Australia). *L. rhamnosus* (LC 705), *L. acidophilus* (74-2), and *Bifidobacterium* spp. (BB 420) were received from Danisco Cultor (Danisco Cultor, Dingley, Australia). *S. thermophilus* (TA040), *L. paracasei* ssp. *paracasei* (LBC81), *L. rhamnosus* (LBA), *L. acidophilus* (LAC 4), and *Bifidobacterium* spp. (BL) were from Rhodia (Rhodia Australia Pty. Ltd., Notting Hill, Australia).

All the strains were tested for purity using Gram stain. All cultures except propionibacteria were propagated weekly in sterile 12% reconstituted skim milk (**RSM**) supplemented with 2% glucose and 1.2% yeast extract. Cultures were grown using 1% inoculum at 37°C for 18 h. Propionibacteria were grown in sodium lactate broth (composition: 10 g of pancreatic digest of casein, 10 g of sodium lactate, 10 g of yeast extract, 0.5 g of Tween 80, and 1 L of distilled water) and incubated at 30°C for 2 d using 1% inoculum. Bifidobacteria were propagated using 1% inoculum in sterile RSM supplemented with 0.05% L-cysteine-hydrochloride in order to provide anaerobic condition and to enhance their growth. Cultures were maintained in the same media at 4°C. Before enumeration the cultures were transferred successively three times for activation.

Media Preparation

Bacteriological peptone and water diluent. Bacteriological peptone and water diluent (0.15%) were prepared by dissolving 1.5 g of bacteriological peptone (Oxoid (Australia) Pty. Ltd., West Heidleberg, Australia) in 1 L of distilled water. The pH was adjusted to 7.0 ± 0.2, followed by autoclaving 9 ml aliquots at 121°C for 15 min.

Streptococcus thermophilus *(ST) agar.* The ST agar was prepared according to the method described by Dave and Shah (1996).

MRS agar, pH-modified (pH 5.20, 4.58) MRS agar, MRS-vancomycine agar, MRS-bile (0.2% and 0.5%) agar, MRS-NaCl agar, and MRS lithium chloride agar. Rehydrated MRS broth (Oxoid) was prepared according to the manufacturer instructions. The pH of the broth was adjusted to 5.20 and 4.58 using 1.0 M HCl to obtain the pH-modified agar. Two and five grams of pure bile salts (Amyl Media, Dandenong Australia)/L were added to obtain 0.2% and 0.5% MRS-bile agar. Forty grams of NaCl/L was added for MRS-NaCl agar (4% final concentration) and 5 g/L lithium chloride (LiCl) was added for MRS-LiCl agar (0.5% final concentration). To prepare MRS-vancomycine (MRS-V) agar, 2 ml of 0.05 g vancomycine (Sigma Chemical Co., Castle Hill, Australia)/100 ml solution was added to 1 L of MRS broth to obtain 1 mg/L final concentration. Agar powder was added to each broth at the rate of 1.2% and the media were autoclaved at 121°C for 15 min. Inoculated plates in duplicates were incubated anaerobically at 37°C and 43°C for 72 h.

MRS-NNLP agar. The MRS-nalidixic acid, neomycine sulfate, lithium chloride and paromomycine sulfate agar (**NNLP**; Sigma Chemical Co.) was prepared according to the method described by Laroia and Martin (1991). MRS agar was the basal medium. Filter-sterilized NNLP was added to the autoclaved MRS base just

Table 1. Viable counts (log_{10} cfu/g) and colony size of bacterial cultures in different sugar-based media (anaerobic incubation, 37°C, 72 h).

	MRS		BA[1]-maltose		BA-galactose		BA-sorbitol		BA-mannitol		BA-esculin	
Cultures	Counts	Size (mm)	Counts	Size (mm)	Counts	Size (mm)	Counts	Size (mm)	Counts	Size (mm)	Counts	Size (mm)
LB-LB 100B	8.28	0.1-0.5	<3.00	—	—	—	<3.00	—	<3.00	—	<3.00	—
ST-TA 040	7.14	0.5 mm	<3.00	—	<3.00	—	<3.00	—	<3.00	—	<3.00	—
ST-DS 2362	8.09	0.5 mm	<3.00	—	<3.00	—	<3.00	—	<3.00	—	<3.00	—
LC-DS 930	9.30	2.0	9.48	2.0	9.30	2.0	9.23	2.0	9.42	2.0	9.21	2.0
LC-LCO 1	9.28	2.0	8.30	2.0	9.15	2.0	9.23	2.0	9.23	2.0	9.21	1.0-1.5
LC-LBC 81	9.07	2.0	9.34	2.0	9.18	2.0	9.28	2.0	9.22	2.0	9.22	1.0-1.5
LR-LC 705	9.20	2.0	9.30	2.0	9.09	2.0	9.18	2.0	9.35	2.0	9.20	1.0-1.5
LR-LBA	9.36	2.0	9.36	2.0	9.04	2.0	9.36	2.0	9.40	2.0	9.15	1.0–1.5
LA-LA 5	7.00	0.1–0.5	8.00	0.5–1	7.70	0.5–1	8.09	0.1–0.5	7.43	0.1–0.5	7.50	0.1–0.5
LA-DS 910	8.41	0.5–1	8.59	1.0–1.5	8.30	1.0–1.5	8.70	0.1–0.5	9.18	0.1–0.5	7.83	0.1–0.5
LA-LAC 4	7.15	0.1–0.5	7.13	0.5–1.0	7.19	.5–1	7.08	0.1–0.5	7.10	0.1–0.5	7.84	0.1–0.5
LA-74-2	7.11	0.1–0.5	8.81	0.5–1.0	7.26	.5–1	8.08	0.1–0.5	7.20	0.1–0.5	7.24	0.1–0.5
BB-Bb 12	<3.00	—	<3.00	—	<3.00	—	<3.00	—	<3.00	—	9.26	1.0–1.5
BB-DS 920	<3.00	—	<3.00	—	<3.00	—	<3.00	—	<3.00	—	8.95	1.0–1.5
BB-420	<3.00	—	<3.00	—	<3.00	—	<3.00	—	<3.00	—	8.88	1.0–1.5
BB-BL	<3.00	—	<3.00	—	<3.00	—	<3.00	—	<3.00	—	<3.00	—
PS-PS 1	10.56	0.1–0.5	<3.00	—	8.11	0.5–1.0	<3.00	—	<3.00	—	<3.00	—
PS-10360	10.84	0.1–0.5	<3.00	—	8.90	0.5–1.0	<3.00	—	<3.00	—	<3.00	—

[1]BA = Basal agar, LB = *L. delbrueckii* ssp. *bulgaricus*, ST = *S. thermophilus*, LC = *L. casei*, LA = *L. acidophilus*, LR = *L. rhamnosus*, BB = *Bifidobacterium*, PS = *Propionibacterium freudenreichii* ssp. *shermanii*.

before pouring. Filter-sterilized L-cysteine-HCl (0.05% final concentration) was also added at the same time to lower the oxidation-reduction potential of the medium and to enhance the growth of anaerobic bifidobacteria. Inoculated plates in duplicates were incubated anaerobically at 37°C for 72 h.

Reinforced clostridial agar. Reinforced clostridial agar (**RCA**; Oxoid) was made according to the manufacturer instructions and sterilized by autoclaving at 121°C for 15 min.

Basal agar, BA-maltose agar, BA-galactose agar, BA-sorbitol agar, BA-mannitol agar, and BA-esculin agar. Basal agar was prepared (composition: 10 g of trypton, 10 g of Lablemco powder, 5 g of yeast extract, 1 g of Tween 80, 2.6 g of K_2HPO_4, 5 g of sodium acetate, 2 g of tri-ammonium citrate, 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.05 g of $MnSO_4 \cdot 4H_2O$, 12 g of bacteriological agar, and 1 L of distilled water) and autoclaved at 121°C for 15 min. Ten milliliters of membrane filtered sterile 20% solutions of maltose, galactose, sorbitol, mannitol or esculin were added to 90 ml of basal agar (2% final concentration) just before pouring the agar medium. Inoculated plates in duplicates were incubated aerobically and anaerobically at 37°C and 43°C for 72 h.

Sodium lactate agar (NaLa agar), arabinose agar, xylose agar, and raffinose agar. The base for these agar media was prepared (composition: 10 g of pancreatic digest of casein, 10 g of yeast extract, 2 g of sodium pyruvate, 2 g of glycine, 1.5 g of sodium chloride, 0.5 g of Tween 80, 0.25 g of di-potasium hydrogen phosphate, 12 g of bacteriological agar and 1 L of distilled water). The pH was adjusted to 7 ± 0.2 using 1 *M* HCl and 10 *M* NaOH. To make NaLa agar, 10 g of sodium lactate was added before autoclaving. The medium was then autoclaved at 121°C for 15 min. For other media, 10 ml of 10% membrane filtered arabinose, raffinose or xylose was added to 90 ml of autoclaved media (1% final concentration) before pouring the plates. Inoculated plates in duplicates were incubated anaerobically at 30°C for 7 to 9 d.

LC agar. LC agar was made using the method described by Ravula and Shah (1998). The incubation was carried out under anaerobic condition at 27°C for 72 h.

Enumeration of Bacteria

Cultures were activated by three successive transfers in nutrient medium before enumeration. One gram of each culture was 10-fold serially diluted (10^3 to 10^7) in 0.15% sterile bacteriological peptone and water diluents. Enumeration was carried out using the pour plate technique. Anaerobic jars and gas generating kits (Anaerobic System, BR 38; Oxoid Ltd., Hampshire, England) were used for creating anaerobic condition. Plates containing 25 to 250 colonies were enumerated and recorded as colony forming units (cfu) per gram of the product or culture.

All experiments and analyses were repeated at least twice. The results presented are averages of at least two replicates.

RESULTS AND DISCUSSION

Viable counts (log_{10} cfu/g) and colony sizes (in mm diameter) of 7 species of bacterial cultures containing 18

strains of bacteria including 1 strain of *L. delbrueckii* ssp. *bulgaricus*, 2 strains of *S. thermophilus*, 3 strains of *L. casei*, 2 strains of *L. rhamnosus*, 4 strains of *L. acidophilus*, 4 strains of *Bifidobacterium* spp., and 2 strains of propionibacteria in various sugar based media are presented in Table 1. *L. delbrueckii* ssp. *bulgaricus* did not grow in any sugar-based media except in MRS agar. MRS agar was particularly suitable for growing lactobacilli. *S. thermophilus* did not grow in any sugar based medium and formed small colonies in MRS agar.

RCA agar supported the growth of all tested organisms. Bifidobacteria grew in this medium even without the addition of L-cysteine.hydrochloride (data not shown). Therefore RCA agar was not suitable for selective enumeration.

ST agar was found to be suitable for *S. thermophilus* (data not shown). *S. thermophilus* formed tiny (0.1–0.5 mm) colonies in ST agar at 37°C under aerobic incubation after 24 h. The incubation time was insufficient for growth of other cultures even if ST agar did not inhibit the growth of other organisms. Therefore, ST agar at 37°C for 24 h and aerobic condition were selective for *S. thermophilus*. This is in agreement with a previous report (Dave and Shah, 1996).

Other organisms such as, *L. casei*, *L. rhamnosus* and *L. acidophilus* grew in all sugar-based media. Bifidobacteria did not grow in any media, except in BA-esculin agar. *Propionibacterium freudenreichii* ssp. *shermanii* grew only in MRS agar and BA-galactose agar. Thus based on sugar utilization patterns, probiotic organisms could not be selectively enumerated.

Table 2 shows the counts of bacterial cultures in media containing different inhibitory substances including vancomycine, NNLP, hydrochloric acid, NaCl, LiCl, and bile at 37°C and 43°C incubations. All the organisms except *Bifidobacterium* spp. grew in MRS agar. When the pH of MRS agar was reduced to 5.20 and the incubation temperature increased to 43°C, only *L. delbrueckii* ssp. *bulgaricus* (which formed 1.0 mm, white rough irregular colonies), *L. rhamnosus* (which formed 2 mm, shiny smooth white colonies) and *L. acidophilus* (which formed 0.1 to 0.5 mm, brown, rough irregular colonies) showed good growth. When the pH of MRS agar was reduced to 4.58 using 1 *M* HCl, only *L. delbrueckii* ssp. *bulgaricus* and *L. rhamnosus* showed good growth similar to that formed in MRS agar at pH 5.20 and the growth of *L. acidophilus* was inhibited except that of DS 910. Therefore, MRS agar at pH 5.20, under anaerobic incubation at 43°C could be selective for *L. delbrueckii* ssp. *bulgaricus* if *L. rhamnosus* and *L. acidophilus* DS 910 were not present in a product. The colony morphology of *L. delbrueckii* ssp. *bulgaricus* and *L. rhamnosus* was very different and these two organisms could be easily differentiated if *L. rhamnosus* was present in the product.

Table 2. Viable counts (log_{10} cfu/g) of bacterial cultures under anaerobic incubation at 37°C and 43°C (LC agar at 27°C) for 72 h in media containing different inhibitory substances.

Cultures	MRS 37°C inc.	MRS-vancomycine 37°C inc.	MRS-vancomycine 43°C inc.	MRS-NNLP 37°C inc.	MRS-pH 5.20 43°C inc.	MRS-pH 4.58 43°C inc.	MRS-NaCl (4%) 37°C inc.	MRS-LiCl (0.5%) 37°C inc.	LC agar 27°C inc.	MRS-bile (0.2%) 43°C inc.	MRS-bile (0.5%) 43°C inc.
LB-LB 100B	9.28	<3.00	<3.00	<3.00	9.37	9.38	<3.00	<3.00	<3.00	<3.00	<3.00
ST-TA 040	7.14	<3.00	<3.00	<3.00	<3.00	<3.00	<3.00	<3.00	<3.00	<3.00	<3.00
ST-DS 2362	8.09	<3.00	<3.00	<3.00	<3.00	<3.00	<3.00	<3.00	<3.00	<3.00	<3.00
LC-DS 930	9.46	9.38	<3.00	<3.00	<3.00	<3.00	9.11	9.14	9.08	<3.00	<3.00
LC-LCO 1	9.37	9.23	<3.00	<3.00	<3.00	<3.00	9.05	9.21	9.09	<3.00	<3.00
LC-LBC 81	9.28	9.22	<3.00	<3.00	<3.00	<3.00	9.26	9.08	9.26	<3.00	<3.00
LR-LC 705	9.20	9.28	9.18	<3.00	9.09	9.08	9.04	9.00	9.45	5.48	<3.00
LR-LBA	9.36	9.37	9.40	<3.00	9.40	9.30	6.36	<3.00	9.00	9.40	7.85
LA-LA 5	7.09	<3.00	<3.00	<3.00	6.48	<3.00	<3.00	<3.00	<3.00	6.04	6.04
LA-DS 910	8.31	<3.00	<3.00	<3.00	8.26	6.89	<3.00	<3.00	<3.00	8.08	<3.00
LA-LAC 4	7.15	<3.00	<3.00	<3.00	6.04	<3.00	<3.00	<3.00	<3.00	6.26	5.04
LA-74-2	7.19	<3.00	<3.00	<3.00	6.04	<3.00	<3.00	<3.00	<3.00	6.10	5.04
BB-Bb 12	<3.00	<3.00	<3.00	7.40	<3.00	<3.00	<3.00	<3.00	<3.00	<3.00	8.78
BB-DS 920	<3.00	<3.00	<3.00	9.18	<3.00	<3.00	<3.00	<3.00	<3.00	<3.00	<3.00
BB-420	<3.00	<3.00	<3.00	7.35	<3.00	<3.00	<3.00	<3.00	<3.00	8.18	<3.00
PS-PS 1	10.54	<3.00	<3.00	<3.00	<3.00	<3.00	<3.00	<3.00	<3.00	<3.00	<3.00
PS-10360	10.84	<3.00	<3.00	<3.00	<3.00	<3.00	<3.00	<3.00	<3.00	<3.00	<3.00

LB = *L. delbrueckii* ssp. *bulgaricus*, LC = *L. casei*, LR = *L. rhamnosus*, LA= *L. acidophilus*, BB = *Bifidobacterium*, PS = *Propionibacterium freudenreichii* ssp. *shermanii*.

Table 3. Viable counts (log_{10} cfu/g) of bacterial cultures in different sugar based media under aerobic incubation at 43°C for 72 h.

Cultures	MRS	BA[1]-sorbitol	BA-manitol	BA-maltose	BA-esculin
LB-LB 180	7.95	<3.00	<3.00	<3.00	<3.00
ST-DS 2362	8.00	<3.00	<3.00	<3.00	<3.00
ST-TA 040	7.69	<3.00	<3.00	<3.00	<3.00
LC-LCO 1	<3.00	<3.00	<3.00	<3.00	<3.00
LC-LBC 81	<3.00	<3.00	<3.00	<3.00	<3.00
LR-LC 705	9.30	9.28	9.24	9.31	9.13
LR-LBA	9.36	9.32	9.30	9.36	9.46
LA-DS 930	<3.00	<3.00	<3.00	<3.00	<3.00
LA-LA 5	6.48	<3.00	<3.00	<3.00	<3.00
LA-DS 910	7.60	<3.00	<3.00	7.08	7.27
LA-74-2	8.93	<3.00	4.00	8.27	8.45
BB-Bb 12	<3.00	<3.00	<3.00	<3.00	<3.00
BB-DS 920	<3.00	<3.00	<3.00	<3.00	<3.00
BB-420	<3.00	<3.00	<3.00	<3.00	<3.00
BB-BL	<3.00	<3.00	<3.00	<3.00	<3.00

[1]Basal agar, LB = *L. delbrueckii* ssp. *bulgaricus*, ST = *S. thermophilus*, LA = *L. acidophilus*, LC = *L. casei*, LR = *L. rhamnosus*, BB = *Bifidobacterium*.

Therefore, pH modified MRS (pH 4.58) agar and anaerobic incubation at 43°C could be used to selectively enumerate *L. delbrueckii* ssp. *bulgaricus* from a product.

L. casei grew in MRS-NaCl (4%), MRS-LiCl (0.5%) at 37°C under anaerobic incubation and LC agar. *L. casei* did not grow in NNLP agar and at 43°C. Lower incubation temperatures (≤37°C) supported the growth of *L. casei*. *L. casei* and *L. rhamnosus* were resistant to 1 mg vancomycine/L. *L. rhamnosus* formed well developed smooth white discs like colonies that were 2 mm or more in diameter in MRS-vancomycine (MRS-V) agar at 37°C under anaerobic incubation. *L. rhamnosus* grew at both incubation temperatures of 37°C and 43°C and in all sugar based media under aerobic and anaerobic conditions, except in MRS-NNLP agar and showed varying growth pattern (between strains) in MRS-bile agar, and MRS-LiCl agar. The organisms grew well in MRS-V agar at both incubation temperatures of 37°C and 43°C as well as in LC agar at 27°C. MRS-V agar at 43°C and anaerobic incubation supported the growth of only *L. rhamnosus*. No other cultures tested including *L. delbrueckii* ssp. *bulgaricus*, *S. thermophilus*, *L. casei*, *L. acidophilus*, *Bifidobacterium lactis* and *Propionibacterium* grew in this medium.

Basal agar (**BA**)-sorbitol agar and BA-mannitol agar under aerobic incubation at 43°C (Table 3) and BA-sorbitol agar and BA-mannitol agar at 43°C and anaerobic incubation (data not shown) also supported the growth of only *L. rhamnosus*. Therefore, MRS-V agar at 43°C under anaerobic incubation, BA-sorbitol agar, or BA-mannitol agar at 43°C, either under aerobic or anaerobic incubations, were selective for *L. rhamnosus*.

MRS-V agar at 37°C or LC agar at 27°C under anaerobic incubation (Table 2) could be selective for *L. casei* when *L. rhamnosus* was not present in a product. When *L. rhamnosus* was present, total counts of *L. casei* and *L. rhamnosus* could be obtained using MRS-V agar at 37°C and anaerobic incubation for 72 h. The count of *L. rhamnosus* on MRS-V agar at 43°C and anaerobic incubation for 72 h could be subtracted from the total counts of *L. casei* and *L. rhamnosus* to obtain the counts of *L. casei*.

MRS-NNLP agar (which contains 0.05% L-cysteine in the formula) at 37°C and anaerobic incubation supported the growth of only bifidobacteria (Table 2). When L-cysteine was not present in the media, bifidobacteria either did not grow or formed pinpoint colonies (data not shown). Therefore, MRS-NNLP agar with 0.05% L-cysteine and anaerobic incubation at 37°C were selective for bifidobacteria and the absence of L-cysteine was able to control the growth of bifidobacteria from other media.

Table 4 shows the colony counts and colony sizes of various bacterial cultures in different agar media. Colonies of ≥0.5 mm in diameter were only counted as developed colonies for the enumeration purpose. NaLa agar, arabinose agar, raffinose agar, and xylose agar supported the growth of *L. casei*, *L. acidophilus*, and *L. rhamnosus* as well as of propionibacteria. In these media, *L. casei* and *L. rhamnosus* formed white shiny smooth colonies of 1 mm diameter.

Propionibacteria formed colonies of 0.5 mm diameter in all the media. However, in NaLa agar, propionibacteria formed colonies that were dull brown with lighter margin of 1.0 to 2.5 mm in diameter. The colonies were very different to those formed by *L. casei* and *L. rhamnosus*. *L. acidophilus* formed pinpoint colonies. To eliminate the possibility of *L. acidophilus* being counted, colonies of ≥0.5 mm diameter were only counted. Proper colonies of propionibacteria formed only after 72 h of incubation and after 7 d colony size grew to 2 mm in

Table 4. Viable counts (log_{10} cfu/g) and colony size of various cultures in different bacteriological media under anaerobic incubation at 30°C (27°C for LC agar) for 7 to 9 d.

Cultures	MRS		NaLa agar		Arabinose agar		Raffinose agar		Xylose agar		LC agar		NaLa agar, day 7 – NaLa agar, day 3[1]
	Count	Colony size (mm)	Count	Colony size (mm)	Count	Colony size (mm)	Count	Colony size (mm)	Count	Colony size (mm)	Count	Colony size (mm)	Count
LC-DS 930	9.46	2.00	9.45	0.5–1.0	9.18	1.0	9.26	1.0	8.30	1.0	9.08	1.5–2.0	<3.00
LC-LC0 1	9.37	2.00	8.78	0.5–1.0	9.18	1.0	9.32	1.0	9.30	1.0	9.10	1.5–2.0	<3.00
LR-LC 705	9.20	2.00	8.86	0.5–1.0	9.23	1.0	9.08	1.0	9.10	1.0	9.35	1.5–2.0	<3.00
LR-LBA	9.14	2.00	8.23	0.5–1.0	8.26	1.0	9.30	1.0	8.43	1.0	9.45	1.5–2.0	<3.00
LA-La 5	7.09	0.1–0.5	6.78	0.1	7.70	0.1	<3.00	—	<3.00	—	<3.00	—	<3.00
LA-DS 910	8.31	0.5–1.0	7.40	0.1	9.08	0.1	<3.00	—	<3.00	—	<3.00	—	<3.00
LA-74-2	7.19	0.1–0.5	6.80	0.1	7.38	0.1	<3.00	—	<3.00	—	<3.00	—	<3.00
BB-Bb 12	<3.00	—	<3.00	—	<3.00		<3.00	—	<3.00	—	<3.00	—	<3.00
BB-DS 920	<3.00	—	<3.00	—	<.300	—	<3.00	—	<3.00	—	<3.00	—	<3.00
PS-PS 1	10.54	0.1–0.5	9.76	1.5–2.0	9.58	1.0	9.75	0.5	9.60	0.5	<3.00	—	9.76
PS-10360	10.84	0.1–0.5	9.90	1.0–2.5	8.66	1.0	9.40	0.5	8.41	0.5	<3.00	—	9.90

[1]Subtraction method (counts in sodium lactate (NaLa) agar at d 7 – counts in NaLa agar at d 3).

LB = *L. delbrueckii* ssp. *bulgaricus*, LA = *L. acidophilus*, LC = *L. casei*, LR = *L. rhamnosus*, BB = *Bifidobacterium*, PS = *Propionibacterium freudenreichii* ssp. *shermanii*.

diameter. The colony sizes of *L. casei* and *L. rhamnosus* did not change. In NaLa agar, the recovery was the highest and colony morphology and colony size were different than those formed in arabinose agar, and xylose agar. Thus, NaLa agar could be used to selectively enumerate propionibacteria. The propionibacteria could also be counted by subtracting the counts of *L. casei* and *L. rhamnosus* at day 3 from the total counts of *L. casei*, *L. rhamnosus* and propionibacteria obtained on d 7 using NaLa agar, raffinose agar or xylose agar (Table 4).

L. acidophilus was found to be the most difficult to enumerate selectively, since most of the media that supported the growth of *L. acidophilus* also supported the growth of *L. casei* and *L. rhamnosus*. When the incubation temperature was increased to 43°C, *L. casei* was eliminated. However, *L. rhamnosus* still formed well developed (1.5 mm in diameter) colonies and *L. acidophilus* formed smaller colonies (0.1 to 1.0 mm) depending on the sugar used (Table 1). When galactose was used, bifidobacteria formed pinpoint colonies in the absence of L-cysteine, and the colonies could be confused with *L. acidophilus*. *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus* formed colonies in MRS agar when incubated anaerobically at 43°C. Therefore, MRS agar and anaerobic incubation at 43°C could be used to enumerate *L. acidophilus* when *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus* were not present in the product (data not shown).

When incubated anaerobically at 43°C in BA-mannitol agar, BA-sorbitol agar, BA-esculin agar and BA-maltose agar, *L. rhamnosus* formed large (2.0 to 2.5 mm diameter) smooth shiny disc like colonies (data not shown), while *L. acidophilus* formed smaller (0.1 to 1.0 mm diameter) rough dull colonies. BA-maltose agar supported the growth of *L. acidophilus* more than the other BA-based agar media at this incubation temperature, but one strain of *L. acidophilus* DS 910 formed large colonies that could be confused with *L. rhamnosus*. In MRS agar, *L. delbrueckii* ssp. *bulgaricus*, *S. thermophilus*, *L. rhamnosus* and *L. acidophilus* formed colonies (Table 3) while in BA-maltose agar, only *L. acidophilus* and *L. rhamnosus* formed colonies. *L. rhamnosus* formed large (2.0 to 2.5 mm diameter) smooth, shiny, and disc like colonies, while strains of *L. acidophilus* formed smaller rough brownish colonies of 0.1 to 1.0 mm diameter that could be easily distinguished. Therefore, MRS agar under aerobic or anaerobic incubation at 43°C could be used to count *L. acidophilus*, except DS 910, when *L. delbrueckii* ssp. *bulgaricus* was not present in the product. If *L. delbrueckii* ssp. *bulgaricus* is present, BA-maltose agar and anaerobic incubation at 43°C could be used and only small rough brownish colonies should be counted as *L. acidophilus*.

Table 5. Media recommended for selective enumeration of *Streptococcus thermophilus*, *Lactobacillus delbrueckii* ssp. *bulgaricus*, *Lactobacillus acidophilus*, *Bifidobacterium*, *Lactobacillus casei*, *Lactobacillus rhamnosus*, and propionibacteria and viable counts of in a mixture of bacteria.

Agar	Bacteria	Incubation conditions	Colony morphology	Counts in a mixture of bacteria (cfu/ml)
S. thermophilus agar	*S. thermophilus*	Aerobic, 37°C, 24 h	0.1–0.5 mm, round yellowish	3.9×10^4
MRS[1] agar (pH 4.58)	*L. delbrueckii* ssp. *bulgaricus*	Anaerobic, 45°C, 72 h	1.0 mm, white, cottony, rough, irregular	7.0×10^7
MRS-sorbitol agar	*L. acidophilus*	Anaerobic, 37°C, 72 h	Rough, dull, small (0.1–0.5), brownish	10.0×10^7
MRS-NNLP[2] agar	Bifidobacteria	Anaerobic, 37°C, 72 h	1 mm, white, smooth, shiny	7.0×10^7
MRS-vancomycine agar[3]	*L. casei*	Anaerobic, 37°C, 72 h	1.0 mm, white shiny, smooth	5.3×10^7
MRS-vancomycine agar	*L. rhamnosus*	Anaerobic, 43°C, 72 h	1.0-2.0 mm, white shiny, smooth	7.6×10^7
Sodium lactate agar	Propionibacteria[4]	Anaerobic, 30°C, 7 to 9 d	1.0–2.5 mm, dull brown, lighter margin	4.9×10^7

[1]deMan, Rogosa, and Sharpe agar.

[2]Nalidixic acid, neomycin sulfate, lithium chloride, and paromomycin sulfate.

[3]If *L. rhamnosus* was not present; however, if *L. rhamnosus* was present, then subtraction methods could be used (i.e., subtracting *L. rhamnosus* counts on MRS-vancomycine agar under anaerobic incubation at 43°C for 72 h from total counts of *L. casei* and *L. rhamnosus* obtained in MRS-vancomycine agar under anaerobic incubation at 37°C for 72 h).

[4]Subtraction method could also be used to determine the counts of propionibacteria (i.e., counts of *L. casei* and *L. rhamnosus* (anaerobic incubation, 30°C, 72 h) could be subtracted from counts of *L. casei*, *L. rhamnosus*, and propionibacteria (anaerobic incubation, 30°C, 7 d).

Among the media tested for *L. acidophilus*, BA-sorbitol agar gave the highest recovery (Table 1). In this medium, *L. casei* and *L. rhamnosus* formed shiny, large, smooth and white colonies, while all strains of *L. acidophilus* tested formed rough dull, small, and brownish colonies. Therefore, only the small dull rough brownish colonies should be enumerated as the counts of *L. acidophilus*.

Table 5 summarizes the media that could be used for selective enumeration of the seven groups of bacteria and their incubation conditions and colony morphology. To verify the efficacy of the method selected in this study, mixtures of *L. delbrueckii* ssp. *bulgaricus*, *S. thermophilus*, *L. acidophilus*, bifidobacteria, *L. casei*, *L. rhamnosus*, and propionibacteria cells were added at approximately 10^7 cfu/ml in the ratio of 0.1, 0.5, 4, 2, 1, 1, and 1, respectively, and the organisms were plated in the media under incubations outlined in Table 5. The identity of each organism was verified by biochemical tests (Kandler and Weiss, 1986). The results are presented in Table 5. As shown in the table, the media were discriminatory for the various groups of bacteria. Thus it appears that the methods could be used for selective enumeration of the seven groups of bacteria used in this study.

Enumeration of Bacteria in Commercial Products

Because the evaluation of media for selective enumeration of yogurt and probiotic bacteria was carried out using pure cultures, it was desirable to validate the efficacy of the method selected using commercial products. Five brands of commercial yogurts and one brand of Swiss cheese were purchased from a local super market, and their bacterial populations analyzed using the different selective bacteriological media. Enumeration of *S. thermophilus* was carried out using ST agar and aerobic incubation at 37°C for 24 h. *L. delbrueckii* ssp. *bulgaricus* was enumerated using MRS-agar (pH 4.58) and anaerobic incubation at 45°C for 72 h. For *L. rhamnosus*, MRS-V agar and anaerobic incubation at 43°C were used. *L. casei* was enumerated using subtraction method, in which viable counts of *L. rhamnosus* on MRS-V agar at 43°C under anaerobic incubation were subtracted from the total counts of *L. casei* and *L. rhamnosus* on MRS-V at 37°C under anaerobic incubation. Bifidobacteria were enumerated on MRS-NNLP agar. Enumeration of *L. acidophilus* was carried out using BA-sorbitol agar at 37°C and anaerobic incubation for 72 h, and BA-maltose agar and anaerobic incubation at 43°C for 72 h. Only the small rough brownish colonies (0.1 to 0.5 mm) were counted as *L. acidophilus*. Propionibacteria were enumerated by subtracting the counts at d 3 of lactic acid bacteria on NaLa agar and anaerobic incubation at 30°C from the total counts of lactic acid bacteria and propionibacteria at day 7.

Table 6 shows the organisms claimed to be present in commercial products and the actual recovery of the organisms. *S. thermophilus* was present in all of yogurts tested. *L. delbrueckii* ssp. *bulgaricus* was present only in product 5 (skinny yogurt). Many commercial products are manufactured using *L. acidophilus*, bifidobacteria and *S. thermophilus* cultures, which do not contain *L. delbrueckii* ssp. *bulgaricus*. *L. casei* was claimed to be present in both products 4 and 5, however, only product 4 (natural yogurt) showed reasonable population of this organism. The stage of shelf life and the pH of yogurt might have affected the viability of the probiotic organism.

Product 2 (natural yogurt) and product 3 (flavored yogurt) had high counts of all organisms claimed including *S. thermophilus*, *L. rhamnosus*, *L. acidophilus*, and

Table 6. Recovery of organisms in commercial products.

Products	Organisms claimed to be present	Organisms present in commercial products						
		L. bulgaricus[1]	*S. thermophilus*[2]	*L. acidophilus*[3]	*L. casei*[4]	*L. rhamnosus*[5]	*B. lactis*[6]	*Propionibacteria*[7]
Product 1 (yogurt)	Yogurt culture	<3.00	8.69	<3.00	<3.00	<3.00	<3.00	<3.00
Product 2 (natural yogurt)	Yogurt culture, *L. acidophilus*, *Bifidobacterium*, and *L. rhamnosus* GG	<3.00	9.17	5.23	<3.00	7.36	7.15	<3.00
Product 3 (flavoured yogurt)	Yogurt culture, *L. acidophilus*, *Bifidobacterium*, and *L. rhamnosus* GG	<3.00	9.01	6.53	<3.00	7.72	7.40	<3.00
Product 4 (natural yogurt)	Yogurt culture, *L. acidophilus*, *Bifidobacterium* and *L. casei*	7.68	8.83	7.83	5.53	<3.00	6.54	<3.00
Product 5 (skinny yogurt)	Yogurt culture, *L. acidophilus*, *Bifidobacterium* and *L. casei*	4.92	8.62	5.51	4.01	<3.00	6.36	<3.00
Product 6 (Swiss cheese)	Cheese culture	<3.00	<3.00	<3.00	<3.00	<3.00	<3.00	3.59

[1]Enumerated using MRS agar (pH 5.58).
[2]Enumerated using *S. thermophilus* agar T agar.
[3]Enumerated using BA-sorbitol agar.
[4]Enumerated using subtraction method (counts in MRS-vancomycine agar at 37°C – counts in MRS-vancomycine agar at 43°C).
[5]Enumerated using MRS-vancomycine agar at 43°C.
[6]Enumerated using MRS-NNLP agar.
[7]Enumerated using subtraction method (counts in NaLa agar at d 7 – counts in NaLa agar at day 3).

bifidobacteria. Products 2 and 3 contained *L. rhamnosus*. Bifidobacteria were found in products 4 and 5 and products 2 and 3 in high concentrations (10^6 to 10^7). *L. acidophilus* also was found in appreciable concentration in all yogurt claimed to contain this organism. Propionibacteria were only found in Swiss cheese (product 6) and this was the only product that claimed to contain propionibacteria. The identity of the organisms was confirmed using Gram stain. Thus it appears that the enumeration methods developed and selected in this study were suitable for enumeration of *S. thermophilus*, *L. delbrueckii* ssp. *bulgaricus*, *L. casei*, *L. rhamnosus*, *L. acidophilus*, bifidobacteria and propionibacteria.

CONCLUSIONS

In this study 19 bacteriological media were evaluated under different incubation conditions for their suitability to recover and enumerate 7 species containing 18 strains of bacteria including *L. delbrueckii* ssp. *bulgaricus*, *S. thermophilus*, *L. casei*, *L. rhamnosus*, *L. acidophilus*, bifidobacteria, and propionibacteria. The evaluation was based on sugar fermentation patterns, use of inhibitory substances (such as acid, bile, salt and antibiotics), different incubation temperatures (27, 30, 37, 43, and 45°C), incubation conditions (such as aerobic and anaerobic) and the duration of incubation (24, 72 h, or 7 to 9 d). ST agar under aerobic incubation at 37°C for 24 h was suitable for *S. thermophilus*. *L. delbrueckii* ssp. *bulgaricus* could be enumerated using MRS agar (pH 4.58) and anaerobic incubation at 45°C for 72 h. MRS-vancomycine agar and anaerobic incubation for 72 h at 43°C were suitable for enumeration of *L. rhamnosus*. MRS-vancomycine agar and anaerobic incubation at 37°C for 72 h or LC agar at 27°C for 72 h and anaerobic incubation were selective for enumeration of *L. casei*, when *L. rhamnosus* was not present in the mixture. *L. casei* could also be enumerated by subtraction method if *L. rhamnosus* was present in the product. The counts of *L. rhamnosus* on MRS-vancomycine agar under anaerobic incubation at 43°C for 72 h could be subtracted from total counts of *L. casei* and *L. rhamnosus* on MRS-vancomycine agar at 37°C for 72 h under anaerobic incubation to obtain *L. casei* count. Bifidobacteria could be enumerated on MRS-NNLP agar. The most suitable method for counting propionibacteria was by subtracting the counts at d 3 of all bacteria except propionibacteria on NaLa agar under anaerobic incubation at 30°C from the total counts at d 7 of all bacteria including propionibacteria under same incubation conditions. Counting large (1.0 to 2.5 mm diameter), smooth brownish colonies with lighter margin on sodium lactate agar after 7 to 9 d at 30°C under anaerobic incubation could also be used to count propionibacteria. *L. acidophilus* could be enumerated on BA-

sorbitol agar at 37°C for 72 h under anaerobic incubation or MRS-agar at 43°C for 72 h under anaerobic incubation or BA-maltose agar at 43°C under anaerobic incubation.

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Survival of *Lactobacillus acidophilus, Lactobacillus paracasei* subsp. *paracasei, Lactobacillus rhamnosus, Bifidobacterium animalis* and *Propionibacterium* in cheese-based dips and the suitability of dips as effective carriers of probiotic bacteria

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Abstract

The suitability of cheese-based dips as a delivery vehicle for probiotic bacteria including *Lactobacillus acidophilus, Lactobacillus paracasei* subsp. *paracasei, Lactobacillus rhamnosus, Bifidobacterium animalis*, and *Propionibacterium freudenreichii* subsp. *shermanii* was studied by evaluating the survival of these organisms in dips. Effects of organic acids, oils and gums, L-cysteine and $NaHCO_3$ on the survival of probiotics in cheese-based dips were also studied. Eight different combinations and five individual bacteria as controls of these probiotic bacteria were added to 21 batches of French onion dip and selective enumeration of these probiotic bacteria was carried out over a period of 10 weeks of storage. The population of *L. acidophilus* and *B. animalis* reduced by 1 log and 2 log per g, respectively. However, when the inoculation level of these bacteria were increased to 8 log per g, they maintained a population of more than 6 log over the shelf life. *L. paracasei* subsp. *paracasei* and *L. rhamnosus* remained at the inoculated level or increased slightly during the storage. A rapid increase in the population of *P. freudenreichii* subsp. *shermanii* occurred to attain more than the inoculation level following reduction in their number by 3 log. Except bacterial interaction, no other factors showed significant effect on the survival of individual probiotic bacteria. Each of *L. acidophilus, B. animalis, L. paracasei* subsp. *paracasei*, and *L. rhamnosus* showed varied levels of antogonism, while *P. freudenreichii* subsp. *shermanii* showed no effect. Any combination of these bacteria can be used as probiotics in cheese-based French onion dip. However, the inoculation level should be 8 log per g for *L. acidophilus* and *B. animalis* and 7 log per g for *L. paracasei* subsp. *paracasei*, and/or *L. rhamnosus* to obtain greater than 6 log of individual bacterial population at the end of shelf life.

Keywords: Probiotics; Cheese-based dips; Survival

1. Introduction

With the emergence of antibiotic resistant bacteria and natural ways of suppressing pathogens, the concept of "probiotics" has attracted much attention. Probiotics are mono- or mixed-cultures of live microorganisms, which when ingested in sufficient numbers, affect the host beneficially by improving the balance of the endogenous micro-flora of the gut (Fuller, 1992). Schaafsma (1996) re-defined 'probiotics' as living organisms that upon ingestion in certain numbers exert health benefit beyond inherent basic nutrition. High levels (at least 10^6 g^{-1}or mL^{-1}) of live microorganisms are recommended for probiotic products (Kurman & Rasic, 1991). A number of therapeutic benefits have been attributed including control of diarrhea, improvement in lactose utilization in lactose malabsorbers, and improvement in host immune responses. The severity of diarrhea in children in day care centers in France was controlled by the consumption of milk fermented with *L. casei* (Pedone, Bernabeu, Postaire, Bouley, & Reinert, 1999). An anti-microbial substance produced by a

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selected strain of *L. acidophilus* was active against *Helicobacter pylori* both in vivo and in vitro (Saavedra, 1995). The production of large quantities of organic acids, and small molecular weight inhibitory substances such as hydrogen peroxide, reuterin, bacteriocins, and competitive exclusion of pathogens by occupying binding sites are some of the mechanisms by which probiotics control the intestinal niche (Shah, 2000). *L. casei*, *B. longum* and *Lactobacillus GG* are found to increase the body's immune response (Perdigon, Demacias, Alvares, Oliver, & Holgado, 1990). Many researchers have observed improved lactose utilization by lactose malabsorbers (Kurman & Rasic, 1991).

The recent trend in food manufacture is to combine probiotics with prebiotics that act as fermentable substrates for probiotics. Prebiotics are not digested by human enzymes but stimulate the growth and/or activity of one or a limited number of bacteria in the colon, thereby improving the host's gut health (Brown, Wang, Topping, Playne, & Conway, 1998). Prebiotics include inulin, lactulose and oligosaccharides (Haesman & Melbintin, 1999). A number of food products including probiotic yoghurt (Kailasapathy & Rybka, 1997), yoghurt like cereal products, drinking yoghurt, power drinks, kefir, ice cream (Haynes & Playne, 2002), frozen fermented dairy deserts (Ravula & Shah, 1998), freeze-dried yoghurt (Rybka & Kailasapathy, 1995), probiotic Cheddar cheese (Stanton et al., 2001), spray dried milk powder (Stanton et al., 2001) fruit and berry juices and coleslaw (Rodgers & Odongo, 2002) have been employed as delivery vehicles for probiotics. Resistant starch has been proven to improve the survival of probiotics in yoghurt (Brown et al., 1998) and in low fat ice cream (Haynes & Playne, 2002). But due to the presence of hydrogen peroxide, high acid levels, inhibitory substances produced by yoghurt bacteria (Shah & Lankaputhra, 1997; Dave & Shah, 1997), high oxygen content (Lankaputhra & Shah, 1997) in the product, injury due to freezing (Lankaputhra, Shah, & Britz, 1996) and freeze drying (Rybka & Kailasapathy, 1995), many of the above-mentioned products have failed to successfully deliver the required level of viable cells of probiotics.

Cheese-based dips could be a delivery vehicle for probiotic bacteria owing to its stable pH, buffering capacity of ingredients used and the presence of prebiotics. At an average consumption of about 50–100 g per serving of dips (Black Swan and Poseidon Dips Pty. Ltd., Victoria, Australia), they can be an effective delivery media for probiotic bacteria, independently or as complementary to other probiotic products. However, little is known about the survival of probiotic bacteria in dips during their shelf life. Ingredients used to improve the texture, safety (pH) and organoleptic qualities of dips such as organic acids (acetic acid, lactic acid and citric acid) and, oil and gums may affect the survival of probiotic bacteria in dips. The potential of improving the microenvironment of the dip in order to improve viability of probiotic bacteria is also worthy of investigation.

The aim of this study was to establish the suitability of cheese-based dips as a delivery vehicle for probiotic bacteria such as *L. acidophilus*, *L. paracasei* subsp. *paracasei*, *L. rhamnosus*, *B. animalis*, and *P. freudenreichii* subsp. *shermanii*. The specific objectives were firstly to identify the best combination of probiotic bacteria that produces maximum cell numbers in dips and then to ascertain the effects of standard dip ingredients such as organic acids, oils and gums and food additives such as L-cysteine and $NaHCO_3$ on the survival of probiotics in dips.

2. Material and methods

2.1. Experimental design and treatments

The study consisted of a sequence of four experiments. The first experiment was designed to select the best combination of five strains of probiotic bacteria in terms of survival in French onion dip. The probiotic bacteria used were:

1. *L. acidophilus* (A)
2. *B. animalis* (B)
3. *L. paracasei* subsp. *paracasei* (C)
4. *L. rhamnosus* (R)
5. *P. freudenreichii* subsp. *shermanii* (P)

A and B were selected because their probiotic properties are well established (Shah, 2004). C and R were selected based on the recent discovery of their probiotic and therapeutic properties (Shah, 2004). Since, C and R are suggested to have similar qualities, they were used mutually exclusively in combinations. P was selected for its capacity to produce vitamin B, which is suggested to improve the quality of the dip and the growth of other probiotic bacteria. The treatments included the five bacteria (as controls) and eight strategic bacterial combinations (ABCP, ABRP, ABC, ABR, BCP, BRP, BC and BR).

The second experiment was designed to determine the effect of type of acid and pH on the survival of probiotic bacteria. The experimental treatments comprised of a factorial combination of three acid types (acetic, lactic and citric acids) and three pH levels (4.45, 4.30 and 4.20). A control treatment with pH 4.45 (legal product requirement) and with a mixture of all three acids (in equal proportions) was included for comparison. Each treatment was replicated twice.

The third experiment determined the effect of addition of canola oil with or without gum (a combination of carboxy methyl cellulose and xanthan gums) to dips on

the survival of probiotic bacteria. The treatments were: oil, oil + gum and control (neither). Each treatment was replicated twice.

The fourth experiment determined the effect of addition of L-cysteine hydrochloride or sodium bicarbonate on the survival of two selected combinations of probiotic bacteria (factorial combinations of two bacterial types and two chemicals).

2.2. Production of the dip

The experimental dip was made according to the formula and methodology adopted by Poseidon and Black Swan Dips, Victoria, Australia for French onion dip. The composition of the dip included (% by weight): cream cheese (62), onion (11), water (20) and minor ingredients (canola oil, lemon juice, vinegar, lactic acid), thickeners and herbs and spices. Immediately after the blending of ingredients, the dip was stored at 4°C before being used in the experiment. For experiments 2–4, a base dip was made without the test material (control). Bacterial cultures and organic acids, oil/gums, L-cysteine hydrochloride or sodium bicarbonate were then added to the base dip.

2.3. Probiotic bacterial cultures

Cultures of *L. acidophilus* (LAC1) and *L. paracasei* subsp. *paracasei* (LCS1) were obtained from DSM (DSM Food Specialties, Australia Pty. Ltd., Werribee, Australia). *B. animalis* (Bb12) and *P. freudenreichii* subsp. *shermanii* (PS1) were received from Chr. Hansen (Chr. Hansen Pty. Ltd. Bayswater, Australia). *L. rhamnosus* (LC 705) was obtained from Bronson and Jacob (Bronson and Jacob, Dingley, Australia). Before use, all organisms were tested for purity using Gram stain and sugar utilization patterns. The starter cultures were in freeze-dried (direct vat set; DVS) form or frozen (DVS) form. The storage and maintenance of the cultures was carried out as per the recommendation of the manufacturers.

2.4. Preparation of media

Bacteriological peptone and water diluent: Bacteriological peptone and water diluent (0.15%) were prepared by dissolving 1.5 g of bacteriological peptone (Oxoid Australia Pty Ltd., West Heidleberg, Australia) in 1 L of distilled water. The pH was adjusted to 7.0 ± 0.2, followed by autoclaving 9 mL aliquots at 121°C for 15 min.

MRS-NNLP agar: The MRS-NNLP (nalidixic acid, neomycine sulfate, lithium chloride and paromomycine sulfate; Sigma Chemical Co. St. Louis, MO) agar was prepared according to the method described by Laroia and Martin (1991). MRS agar was the basal medium. Filter sterilized NNLP was added to the autoclaved MRS base just before pouring. Filter sterilized L-cysteine·HCl (0.05% final concentration) was also added at the same time to lower the oxidation–reduction potential of the medium and to enhance the growth of anaerobic bifidobacteria. Inoculated plates in duplicates were incubated at 37°C anaerobically for 72 h.

MRS-vancomycin agar, MRS-sorbitol agar, sodium lactate (NaLa) agar: The MRS-vancomycin agar, MRS-sorbitol agar and sodium lactate agar were prepared according to Tharmaraj and Shah (2003).

2.5. Enumeration of bacteria

Ten grams of dip was mixed with 90 mL of 0.15% sterile bacteriological peptone followed by mixing homogeneously using a stomacher and 10-fold serial dilution (10^3–10^7) were prepared. The enumeration was carried out using the pour plate technique. Duplicate plates were incubated anaerobically at 37°C for 72 h in a gas mixture of 10% CO_2, 5% H_2 and 85% N_2 in anaerobic jars using gas generating kits (Anaerobic System BR 38; Oxoid Ltd., Hampshire, England). Plates containing 25–250 colonies were enumerated and recorded as colony forming units (cfu) gram^{-1}of the product or culture. The enumeration of *L. acidophilus*, *L. paracasei* subsp. *paracasei*, *L. rhamnosus* and *P. freudenreichii* subsp. *shermanii* was carried out as described by Tharmaraj and Shah (2003). *L. acidophilus*, *L. paracasei* subsp. *paracasei* and *L. rhamnosus* were enumerated on MRS-sorbitol agar. Colonies that were irregular in shape with a diameter of 0.1–0.5 mm were counted as *L. acidophilus*. White smooth shiny disc-like colonies with a diameter of 1–2 mm were counted as of *L. paracasei* subsp. *paracasei* or of *L. rhamnosus*. *L. paracasei* subsp. *paracasei* and *L. rhamnosus* were also enumerated on MRS-vancomycin agar. Since *L. paracasei* subsp. *paracasei* and *L. rhamnosus* have not been added together in any of the combinations, the subtraction method described by Tharmaraj and Shah (2003) have not been used. *B. animalis* was counted on NNLP agar as described by Laroia and Martin (1991) and *P. freudenreichii* subsp. *shermanii* was counted on NaLa (sodium lactate) agar. Brownish smooth shiny lenticulate colonies with a diameter of 1–3 mm were counted as *P. freudenreichii* subsp. *shermanii*.

2.6. Experiment 1

Survival of probiotic bacteria in dip: The different bacterial consortium (8 combinations + 5 controls), in two replicates, was inoculated to 2.5 kg lots of French onion dips at a level of log 7 cfu g^{-1} (notionally) and mixed well aseptically in a laboratory mixer. The dips (26 types) were then packed in 150 g portions in non-transparent plastic containers, sealed airtight and stored

at 4°C for a period of 10 weeks. Duplicate samples were collected from each dip at 2 weeks interval. The duplicate samples from each replicate were bulked, mixed homogeneously and a sub-sample was aseptically taken for microbiological count. pH was measured on the rest of the samples.

2.7. Experiment 2

Effect of pH and type of organic acids on the survival of probiotic bacteria: From the results of the first experiment, the bacterial combination ABCP was selected for this experiment. Initially, a base dip was prepared as outlined before omitting acids. The four bacterial cultures (*L. acidophilus* (A), *B. animalis* (B), *L. paracasei* subsp. *paracasei* (C) and *P. freudenreichii* subsp. *shermanii* (P)) were inoculated at a rate of 10^7 cfu g^{-1} in each of the 10 batches of dips (three acid types × three pH levels + control). Citric acid (10.0, 15.0 and 20.0 mL of 10% solution of citric acid kg^{-1}) acetic acid (2.0, 3.0 and 4.0 mL of acetic acid kg^{-1}) or lactic acid (1.25, 2.0 and 2.5 mL of lactic acid kg^{-1}) were added separately to bring the pH to three different levels (4.45, 4.30 and 4.20). The probiotic dips were mixed homogeneously, and packed and sealed airtight in 150 g portions. The sealed containers were stored at 4°C for a period of 10 weeks. Starting from day 2, duplicate samples were collected at 2-week intervals for 10 weeks, from each batch of dip for analysis. Duplicate samples from each replicate of each replicate were mixed homogeneously and a sub-sample was aseptically taken for microbiological analysis. The rest of the samples were used to measure pH.

2.8. Experiment 3

Effect of oil and gums on the survival of probiotic bacteria: Initially, 5 kg of French onion dip was made without oil and gums. Canola oil or gum was added to the base dip and four bacterial cultures consisting of ABCP (*L. acidophilus* (A), *B. animalis* (B), *L. paracasei* subsp. *paracasei* (C) and *P. freudenreichii* subsp. *shermanii* (P)) were inoculated at a rate of 10^7 cfu g^{-1}, mixed homogeneously, and packed and sealed airtight in 150 g portions. The sealed containers were stored at 4°C for a period of 10 weeks. Starting from day 2, duplicate samples were collected at 2-weeks intervals for 10 weeks, from each treatment for analysis. The duplicate samples of each treatment were mixed homogeneously and a sub-sample was aseptically taken for microbiological count.

2.9. Experiment 4

Effect of l-cysteine hydrochloride and sodium bicarbonate on the survival of probiotic bacteria: Since *P. freudenreichii* subsp. *shermanii* was found to survive well in dips in all of the earlier experiments, this organism was not selected for this experiment. The combinations ABC (*L. acidophilus* (A), *B. animalis* (B), *L. paracasei* subsp. *paracasei* (C)) and ABR (*L. acidophilus* (A), *B. animalis* (B), *L. rhamnosus* (R)) were used in this study. L-cysteine was added to reduce the oxidation–reduction potential of the dip. Sodium bicarbonate was added to neutralize the acid effect and to produce HCO_3^- and CO_2. The additives, were mixed with the dip at the rate of 0.05% by weight. Each of the two types of probiotic dips (ABC and ABR) was prepared by mixing the respective bacterial cultures to French onion dip and packed in 150 g portions in plastic containers. The sealed containers were stored at 4°C for 10 weeks. Enumeration of bacteria was performed at day 2 (week 0), week 2, week 4, week 6, week 8, and week 10.

2.10. Statistical analysis

The results were analysed by general analysis of variance model using the GENSTAT program (Genstat committee, 1995). In experiment 1, each bacterial type was analysed and presented separately for differences between individual bacterial type (control) and its combinations. Means were compared using the least significant difference (LSD). In experiment 2, only the data belonging to the three types of acids with pH 4.45 and control was subjected to statistical analysis.

3. Results and discussions

3.1. Effects of bacterial combinations on the survival of probiotic bacteria in dips

The changes in bacterial population over 10 weeks of refrigerated storage of *L. acidophilus*, *B. animalis*, *L. paracasei* subsp. *paracasei*, *L. rhamnosus* and *P. freudenreichi* subsp. *shermanii* are given in Tables 1–5. Since the number of combinations of each of the five bacteria was different, the data of each bacterium was analysed and presented separately. When the average counts of individual bacterial types (the 5 control treatments) were compared, the order of bacterial type in terms of survival over 10 weeks period ($P<0.001$) was *L. paracasei* subsp. *paracasei* > *L. rhamnosus* > *P. freudenreichi* subsp. *shermanii* > *L. acidophilus* = *B. animalis* (Tables 1–5). The survival of *L. acidophilus* (A) in control (log 6.3) was less than that of combinations, but was not significantly ($P>0.05$) affected by the bacterial combination (log 6.5–6.7) (Table 1). However, irrespective of the bacterial combination, the population of *L. acidophilus* declined over storage at an average rate of 0.01 log unit per day,

and lost about 0.9 log population over 10 weeks. Initially, the population dropped by 11.7% in 2 days and then at a slow rate of around 0.9% per 2 weeks. The viable population of 6.35 log g^{-1} after 10 weeks resulted from an initial inoculation rate of 7.39 log g^{-1}. Since dips are consumed at relatively smaller quantities (50–100 g serving^{-1}), a higher inoculation rate (around 9 log g^{-1}) of *L. acidophilus* may be needed to maintain

Table 1
Changes in $\log_{10}$ population (cfu g^{-1}) of *Lactobacillus acidophilus* in dips with different culture combinations over 10 weeks of storage

Time in shelf (weeks)	Bacterial combination				
	ABCP	ABRP	ABC	ABR	A (Control)
0 (initial)	7.4	7.4	7.4	7.4	7.4
1	6.5	6.4	6.6	6.6	6.5
2	6.5	6.5	6.7	6.8	6.4
4	6.4	6.3	6.6	6.5	6.1
6	6.3	6.4	6.5	6.5	6.0
8	6.3	6.5	6.4	6.4	6.0
10	6.4	6.4	6.4	6.2	5.8
Average	6.5^{a}	6.6^{a}	6.7^{a}	6.6^{a}	6.3^{b}
SED	0.068				
$LSD_{0.05}$	0.14**				

SED = Standard error difference of mean.
$LSD_{0.05}$ = Least significant difference at $P<0.05$.
ABCP—*L. acidophilus*, *B. animalis*, *L. paracasei* subsp. *paracasei*, *P. freudenreichii*.
ABRP—*L. acidophilus*, *B. animalis*, *Lactobacillus rhamnosus*, *P. freudenreichii*.
ABC—*L. acidophilus*, *B. animalis*, *L. paracasei* subsp. *paracasei*.
ABR—*L. acidophilus*, *B. animalis*, *L. rhamnosus*.
A—*L. acidophilus*.
** Means significantly different at $P<0.001$. Means with different superscripts within row differ significantly at $P<0.05$.

Table 3
Changes in $\log_{10}$ population (cfu g^{-1}) of *Lactobacillus paracasei* subsp. *paracasei* in dips with different bacterial combinations over 10 weeks of storage

Storage time (weeks)	Bacterial combination				
	ABCP	ABC	BCP	BC	C (Control)
0 (initial)	7.4	7.4	7.4	7.4	7.4
1	7.1	6.8	6.8	6.9	7.1
2	7.3	7.7	7.8	6.8	7.4
4	7.2	7.7	7.6	7.7	7.5
6	8.1	7.9	7.7	7.7	8.1
8	8.1	8.0	7.9	7.9	8.4
10	7.7	7.8	6.5	7.8	8.5
Average	7.6^{b}	7.6^{b}	7.4^{c}	7.5^{bc}	7.8^{a}
SED	0.071				
$LSD_{0.05}$	0.14**				

SED = Standard error difference of mean.
$LSD_{0.05}$ = Least significant difference at $P<0.05$.
ABCP—*L. acidophilus*, *B. animalis*, *L. paracasei* subsp. *paracasei*, *P. freudenreichii*.
ABC—*L. acidophilus*, *B.animalis*, *L. paracasei* subsp. *paracasei*.
BCP—*B. animalis*, *L. paracasei* subsp. *paracasei*, *P. freudenreichii*.
BC—*B. animalis*, *L. paracasei* subsp. *paracasei*.
C—*L. paracasei* subsp. *paracasei*.
** Means significantly different at $P<0.001$. Means with different superscripts within row differ significantly at $P<0.05$.

Table 2
Changes in $\log_{10}$ population (cfu g^{-1}) of *Bifidobacterium animalis* in dips with different bacterial combinations over 10 weeks of storage

Storage time (weeks)	Bacterial combination								
	ABCP	ABRP	ABC	ABR	BCP	BRP	BC	BR	B (Control)
0 (initial)	7.4	7.4	7.4	7.4	7.4	7.4	7.4	7.4	7.4
1	6.4	6.5	6.6	6.6	5.7	5.7	5.9	5.7	6.9
2	6.5	6.5	6.6	6.7	5.7	5.8	5.8	5.7	6.6
4	6.5	6.4	6.5	6.5	5.7	5.8	5.8	5.4	6.5
6	5.9	6.0	5.9	6.0	5.9	6.0	5.9	5.8	6.1
8	6.0	5.8	5.8	5.8	5.9	6.0	5.8	5.4	5.5
10	5.7	5.5	5.6	5.5	5.6	6.0	5.8	5.4	5.0
Average	6.4^{a}	6.3^{ab}	6.4^{a}	6.4^{a}	6.0^{d}	6.1^{c}	6.0^{c}	5.9^{e}	6.3^{b}
SED	0.035								
$LSD_{0.05}$	0.07**								

SED = Standard error difference of mean.
$LSD_{0.05}$ = Least significant difference at $P<0.05$.
ABCP—*L. acidophilus*, *B. animalis*, *L. paracasei* subsp. *paracasei*, *P. freudenreichii*.
ABRP—*L. acidophilus*, *B. animalis*, *L. rhamnosus*, *P. freudenreichii*.
ABC—*L. acidophilus*, *B.animalis*, *L. paracasei* subsp. *paracasei*.
ABR—*L. acidophilus*, *B. animalis*, *L. rhamnosus*.
BCP—*B. animalis*, *L. paracasei* subsp. *paracasei*, *P. freudenreichii*.
BRP—*B. animalis*, *L. rhamnosus*, *P. freudenreichii*.
BC—*B. animalis*, *L. paracasei* subsp. *paracasei*.
BR—*B. animalis*, *L. rhamnosus*.
B—*B. animalis*.
** Means significantly different at $P<0.001$. Means with different superscripts within row differ significantly at $P<0.05$.

Table 4
Changes in $\log_{10}$ population (cfu g^{-1}) of *Lactobacillus rhamnosus* in dips with different bacterial combinations over 10 weeks of storage

Storage time (weeks)	Bacterial combination				
	ABRP	ABR	BRP	BR	R (Control)
0 (initial)	7.4	7.4	7.4	7.4	7.4
1	7.5	7.5	7.4	7.3	7.4
2	7.4	7.7	7.5	7.3	7.4
4	7.5	7.4	7.4	7.7	7.6
6	7.5	7.5	7.3	7.2	7.8
8	7.6	7.4	7.3	7.5	8.0
10	7.6	7.0	7.0	7.2	8.1
Average	7.5^{ab}	7.4^{b}	7.3^{c}	7.4^{b}	7.6^{a}
SED	0.073				
$LSD_{0.05}$	0.15**				

SED = Standard error difference of mean.
$LSD_{0.05}$ = Least significant difference at $P<0.05$.
ABRP—*L. acidophilus*, *B. animalis*, *L. rhamnosus*, *P. freudenreichii*.
ABR—*L. acidophilus*, *B. animalis*, *L. rhamnosus*.
BRP—*B. animalis*, *L. rhamnosus*, *P. freudenreichii*.
BR—*B. animalis*, *L. rhamnosus*.
R—*L. rhamnosus*.
** Means significantly different at $P<0.001$. Means with different superscripts within row differ significantly at $P<0.05$.

Table 5
Changes in $\log_{10}$ population (cfu g^{-1}) of *Propionibacterium freudenreichii* subsp. *shermanii* in dips with different bacterial combinations over 10 weeks of storage

Storage time (weeks)	Bacterial combination				
	ABCP	ABRP	BCP	BRP	P (Control)
0 (initial)	7.4	7.4	7.4	7.4	7.4
1	<4.0	<4.0	<4.0	<4.0	6.0
2	<4.0	<4.0	<4.0	<4.0	6.1
4	4.3	4.3	<4.0	<4.0	6.5
6	5.6	6.0	6.0	5.4	7.0
8	6.0	6.0	6.1	6.6	7.2
10	6.5	6.5	6.5	6.5	7.3
Average	5.4^{b}	5.5^{b}	5.4^{b}	5.4^{b}	6.8^{a}
SED	0.09				
$LSD_{0.05}$	0.09**				

SED = Standard error difference of mean.
$LSD_{0.05}$ = Least significant difference at $P<0.05$.
ABCP—*L. acidophilus*, *B. animalis*, *L. paracasei* subsp. *paracasei*, *P. freudenreichii*.
ABRP—*L. acidophilus*, *B. animalis*, *L. rhamnosus*, *P. freudenreichii*.
BCP—*B. animalis*, *L. paracasei* subsp. *paracasei*, *P. freudenreichii*.
BRP—*B. animalis*, *L. rhamnosus*, *P. freudenreichii*.
P—*P. freudenreichii*.
** Means significantly different at $P<0.001$. Means with different superscripts within row differ significantly at $P<0.05$.

and deliver the beneficial population levels for 10 weeks. Although, the survival of *L. acidophilus* did not vary significantly between bacterial combinations, the combinations without *P. freudenreichi* subsp. *shermani* (ABC and ABR) contained greater populations of *L. acidophilus* compared to those with this organism (ABCP and ABRP). The results also indicate that *L. acidophilus* survives better in the presence of *L. paracasei* subsp. *paracasei* than *L. rhamnosus*, and that ABC or ABCP provided the best bacterial combination for the survival of *L. acidophilus*.

The survival of *B. animalis* (B) was significantly ($P<0.01$) affected by the bacterial combination and during storage ($P<0.01$) (Table 2). The population of *B. animalis* dropped by about 1 log in the first week, remained static until week 4 and thereafter declined slowly by about 0.8–1.0 log between week 4 and week 10. However, there was a significant ($P<0.01$) interaction between bacterial type and storage days in the changes in *B. animalis* population. In all four combinations in which *L. acidophilus* was present, the initial drop in the population of *B. animalis* was relatively smaller than that in combinations without *L. acidophilus*. In the absence of *L. acidophilus*, the population of *B. animalis* dropped well below the critical level of log 6 cfu g^{-1} (Kurman & Rasic, 1991) within the first week (Table 2). In combinations with *L. acidophilus*, the population of *B. animalis* remained above the lower critical level for up to 6 weeks. Out of the four bacterial combinations that contained *L. acidophilus*, the survival of *B. animalis* was highest in ABCP combination during the 10-week storage. Out of all combinations, the count of *B. animalis* was the least in BR. All combinations of *B. animalis* with *L. paracasei* subsp. *paracasei* contained almost similar *B. animalis* population at the end of 10 weeks (Table 2). A reduction in log population of 1.55–1.70 g^{-1} to that of the initial population was found in these combinations, whereas the combinations with *L. rhamnosus* showed reduction of up to 2 log cycles g^{-1}. The presence of *P. freudenreichii* subsp. *shermanii* did not appear to affect the final population at 10 weeks. These findings indicate that *L. paracasei* subsp. *paracasei* and *L. rhamnosus* might inhibit *B. animalis*, while the effect of *L. rhamnosus* might be more than that of *L. paracasei* subsp. *paracasei*. These findings were further confirmed by the experiments carried out on the inhibitory effects within probiotics (data not shown). However, in BRP and BC combinations, after a drastic initial drop, the population of *B. animalis* remained static throughout the 10 weeks storage period. This suggests that at a higher initial inoculation level (log 9–10 g^{-1}), *B. animalis* may survive above required levels for a longer time.

The bacterial strains *L. paracasei* subsp. *paracasei* (C) and *L. rhamnosus* were observed to be affected positively to produce populations that are greater than that of the inoculation rate. Similar pattern was reported to be observed elsewhere with another strain of *L. paracasei* subsp. *paracasei* strain LBC81 (Lane, 2000). Survival of *L. paracasei* subsp. *paracasei* (C) was significantly affected by the bacterial combination ($P<0.05$) and

days on shelf ($P<0.01$, Table 3). Though the population of *L. paracasei* subsp. *paracasei* declined at day 2, thereafter, it increased during 10 weeks storage. The population growth pattern of *L. paracasei* subsp. *paracasei* varied significantly ($P<0.01$) with time between bacterial combinations (Table 3). In ABC and BCP combinations, *L. paracasei* subsp. *paracasei* started to grow above the initial level by week 2 but thereafter remained relatively static until week 10. In ABCP, the population of *L. paracasei* subsp. *paracasei* remained relatively static from week 0 to week 4 and thereafter increased by about one log cycle g^{-1}. This increase in the population of *L. paracasei* subsp. *paracasei* was greater in the ABC combination than ABCP one. The increase in *L. paracasei* subsp. *paracasei* population remained after week 6 in ABCP combination. In contrast, the population of *L. paracasei* subsp. *paracasei* declined below the initial level in BCP combination at week 10. The difference in the survival of *L. paracasei* subsp. *paracasei* in ABCP and BCP and ABC and BC combinations (Table 3) is suggestive of a beneficial effect of *L. acidophilus* on the growth and survival of *L. paracasei* subsp. *paracasei*. However, at the end of week 10, ABC and BC showed the highest population level of *L. paracasei* subsp. *paracasei*, nullifying the effect of *L. acidophilus*. When inoculated at a rate of 7.40 log g^{-1}, all the combinations contained well above the required population of log 6 cfu g^{-1} of *L. paracasei* subsp. *paracasei* throughout the storage.

Table 4 shows the survival of *L. rhamnosus* (R) population in four different bacterial combinations. The bacterial combination or storage did not significantly affect the survival of *L. rhamnosus* ($P>0.05$).

The survival of *P. freudenreichii* subsp. *shermanii* (P) was not affected by the bacterial combination (Table 5). From week 0 to week 2–4, the population of this bacterium declined below 4 log units. Thereafter, *P. freudenreichii* subsp. *shermanii* started to grow steadily till the end of 10 weeks storage. This is in line with technical data provided by the starter culture supplier. Certain organisms are reported to grow at refrigerated temperature (4°C) and cause post-acidification (Shah, 2000). This may indicate that *P. freudenreichii* subsp. *shermanii* is able to grow or recover from the injury initially caused by the dip condition, at refrigeration temperature and might either have grown some resistance to conditions prevailed in the dip that suppressed or inhibited initially. *P. freudenreichii* subsp. *shermanii* started to grow relatively earlier (week 4) in the presence of *L. acidophilus* in the combination (ABCP and ABRP) than in combinations without *L. acidophilus* (BCP and BRP). Further investigation is needed to establish the reason for the initial drastic drop in their populations.

Table 6 shows the rank of survival of each type of bacteria in different bacterial combinations. *L. acidophilus* (A) survived the best in the combination ABC followed by ABCP. *B. animalis* (B) survived the best in combination ABCP followed by ABC. Bacterial combinations did not affect *P. freudenreichii* subsp. *shermanii* (P). The combination ABC performed the best followed by ABCP, ABRP and ABR when *L. paracasei* ssp. *paracasei* (C) and *L. rhamnosus* (R) considered together. From the above results, the combination ABC could be selected as the best combination where the probiotics (*L. acidophilus* (A), *B. animalis* (B) and *L. paracasei* subsp. *paracasei* (C) performed the best. The combination ABCP (*L. acidophilus* (A), *B. animalis* (B) and *L. paracasei* subsp. *paracasei* (C) and *P. freudenreichii* subsp. *shermanii* (P) could be selected as the second best. These results suggest that *L. acidophilus* is contributing positively to the survival of *B. animalis*, *L. paracasei* subsp. *paracasei* and *L. rhamnosus*. *B. animalis* appeared to be antagonistic to *L. paracasei* subsp. *paracasei* and *L. rhamnosus*. *B. animalis* and *P. freudenreichii* subsp. *shermanii* appeared to have a negative effect on *L. paracasei* subsp. *paracasei* and *L. rhamnosus*. This negative effect appears to be additive as in the presence of both *B. animalis* and *P. freudenreichii* subsp. *shermanii*, the survival rank (Table 6) of the combinations of BCP (*L. paracasei* subsp. *paracasei*, *B. animalis* and *P. freudenreichii* subsp. *shermanii*) and BRP (*L. rhamnosus*, *B. animalis* and *P. freudenreichii* subsp. *shermanii*) were pushed to the last. The positive effect of *L. acidophilus* on the other bacteria appears to be strong to overpower and nullify the antagonistic effects of *B. animalis* on *L. rhamnosus* and *L. paracasei* subsp. *paracasei*. The antagonistic effect of *B. animalis* and *L. rhamnosus* and/or *L. paracasei* subsp. *paracasei* appears to be mutual (Table 2). The population of

Table 6
Ranks of survival rate of *Lactobacillus acidophilus*, A; *Bifidobacterium animalis*, B; *Lactobacillus paracasei* subsp. *paracasei*, C and *Propionibacterium freudenreichii* subsp. *shermanii*, P in different probiotic combinations

Bacteria	Rank of bacterial combination for each type of bacteria							
	ABCP	ABRP	ABC	ABR	BCP	BRP	BC	BR
A	2	3	1	4				
B	1	2	2	2	4	3	3	4
C	2		1		4		3	
R		1		2		4		3
C/R	2	3	1	4	7	8	5	6
P	1	1			1	1		

ABCP—*L. acidophilus*, *B. animalis*, *L. paracasei* subsp. *paracasei*, *P. freudenreichii*.
ABRP—*L. acidophilus*, *B. animalis*, *L. rhamnosus*, *P. freudenreichii*.
ABC—*L. acidophilus*, *B. animalis*, *L. paracasei* subsp. *paracasei*.
ABR—*L. acidophilus*, *B. animalis*, *L. rhamnosus*.
BCP—*B. animalis*, *L. paracasei* subsp. *paracasei*, *P. freudenreichii*.
BRP—*B. animalis*, *L. rhamnosus*, *P. freudenreichii*.
BC—*B. animalis*, *L. paracasei* subsp. *paracasei*.
BR—*B. animalis*, *L. rhamnosus*.

B. animalis was the least in combinations of BC (*B. animalis* and *L. paracasei* subsp. *paracasei*) and BR (*B. animalis* and *L. rhamnosus*) indicating that *L. paracasei* subsp. *paracasei* and *L. rhamnosus* have affected the population of *B. animalis* negatively. The antagonistic effects of these probiotic bacteria need further investigation. *P. freudenreichii* subsp. *shermanii* did not appear to interfere with any of the other bacteria.

3.2. Effect of pH and type of organic acid on the survival of probiotic bacteria

Lactic acid, acetic acid and citric acid are naturally occurring and most commonly used organic acids to enhance organoleptic qualities as well as safety of many food products. Lactic acid bacteria are found to be more tolerant to acidity and organic acids than most of the pathogens and spoilage microorganisms. The effect of organic acids at different pH levels of 4.45, 4.30, 4.20 on the population of probiotic bacteria is shown in Table 7. On average, *L. paracasei* subsp. *paracasei* (6.6 $\log g^{-1}$) and *P. freudenreichii* subsp. *shermanii* (6.7 $\log g^{-1}$) survived better than *L. acidophilus* (5.9 $\log g^{-1}$) and *B. animalis* (5.8 $\log g^{-1}$) over the 10 weeks of storage period.

L. acidophilus and *B. animalis* were not significantly affected at pH levels of 4.45, 4.30 and 4.20 in any of the tested organic acids after the initial reduction of 1 $\log g^{-1}$ at the end of 2 weeks. The initial reduction might be due to the initial higher temperature and acidity of the product or due to the antagonism among probiotic bacteria, while the metabolic activity was higher before the product reached the storage temperature of 4°C (Table 7). It has been reported that *L. acidophilus* and *B. animalis* are affected by pH 5.0 (Shah, Lankaputhra, Britz, & Kyle, 1995; Lankaputhra & Shah, 1997). The information on antagonistic effects among *L. acidophilus*, *B. animalis*, *L. paracasei* subsp. *paracasei* and *P. freudenreichii* subsp. *shermanii* is limited. After 2 weeks, the level of reduction with acetic acid was higher than the other two acids for *L. acidophilus*. *B. animalis* performed better in acetic acid than in the other acids. This might be due to the reduced antagonistic effect of the other bacteria that were inhibited by acetic acid or *B. animalis* might be more resistant to acetic acid than other bacteria since acetic acid is one of the metabolites of this bacterium.

L. paracasei subsp. *paracasei* was not adversely affected by any acids or at any pH levels, and the organisms showed an increase in the population of 0.04–0.89 log in lactic and citric acid at the pH of 4.45–4.20. In acetic acid, this bacterium showed varied levels of reduction in their population. Acetic acid might have inhibited *L. paracasei* subsp. *paracasei* slightly (Table 7). This inhibitory effect of acetic acid on *L. paracasei* subsp. *paracasei* might be the reason for the effect of *B. animalis* observed in the earlier experiment (Table 3). But *L. paracasei* subsp. *paracasei* performed slightly better in pH 4.20 than in pH of 4.45. This might be due to the antagonistic effect of co-bacteria that tolerated acetic acid slightly better than *L. paracasei* subsp. *paracasei*, possibly *B. animalis*. At pH 4.30, the antagonistic co-bacteria might have lost their tolerance to acetic acid and control over *L. paracasei* subsp. *paracasei*, allowing it to show better survival. At pH 4.20, *L. paracasei* subsp. *paracasei* appeared to have been affected by acetic acid. The overall excellent survival of *L. paracasei* subsp. *paracasei* might indicate that *L. paracasei* subsp. *paracasei* is resistant to higher acid levels or the organism did not suffer bacterial antagonism or dominated the niche by suppressing other probiotic bacteria present. This speculation needs to be verified.

P. freudenreichii subsp. *shermanii* population observed to have reduced by 2 log cycle by the end of week 2 but after week 2 this organism showed continuous growth till the end of the storage. This effect was similar to that of Table 5. In all treatments (Table 7), the initial suppression of *P. freudenreichii* subsp. *shermanii* might be due to bacterial antagonism during the high metabolic activity of co-bacteria during the early incubation period. The population growth after week 2 might indicate the ability of *P. freudenreichii* subsp. *shermanii* to grow and proliferate at lower storage temperatures and at lower pH levels, where the metabolic activities of other bacteria are at their minimal.

Table 7 shows that the treatment control, which had all three organic acids and a pH of 4.45 supported all probiotic bacteria better than single acid treatments. Antimicrobial property of acids depends on temperature, pK, concentration and pH along with its lipophilic property and solubility. Acetic acid is the most lipophilic out of the three acids. pK is the pH at which concentrations of the undissociated molecules and the dissociated molecules are equal. The pK values of acetic, lactic and acids citric are 4.80, 3.86 and 3.06, respectively. The acetic acid with higher pK value might have had higher proportion of undissociated molecules that are more lipophilic and antimicrobial. This might be another reason for the suppression of probiotics shown in the combinations with acetic acid. When the concentration of acetic acid was reduced in the control dip by the inclusion of other acids (acetic acid: lactic acid: citric acid = 1:1:1), though the pH was similar, the suppressive effect was reduced. The drastic reduction observed during the first 2 weeks might be due to the temperature effect with acid effect, where the temperature dropped from room temperature to 4°C. At room temperature, the suppression effect of acids might have been more than at 4°C.

The pH of the experimental dips over a period of 10 weeks is shown in Fig. 1. In all the treatments, the pH

Table 7
Effect of acid type and initial pH on log 10 population (cfu g^{-1}) of *Lactobacillus acidophilus*, *Bifidobacterium animalis*, *Lactobacillus paracasei* subsp. *paracasei* and *Propionibacterium freudenreichii* subsp. *shermanii* over 10 weeks of storage

Time (weeks)	Organic acid type/ Initial pH									
	Lactic acid			Citric acid			Acetic acid			Control (all 3 acids)
	4.45	4.30	4.20	4.45	4.30	4.20	4.45	4.30	4.20	4.45
Lactobacillus acidophilus										
0	6.4	6.4	6.4	6.4	6.4	6.4	6.4	6.4	6.4	6.4
2	6.0	5.7	5.5	5.7	5.5	5.3	5.2	5.2	5.1	5.9
4	5.2	5.2	5.1	5.3	5.1	5.1	5.2	5.0	5.0	5.3
6	5.3	5.2	5.2	5.3	5.1	5.2	5.2	5.1	5.1	5.5
8	5.3	5.2	5.2	5.4	5.2	5.1	5.1	5.1	5.1	5.8
10	5.5	5.1	5.2	5.3	5.2	4.4	5.1	4.9	4.0	5.5
Average	5.6	5.5	5.4	5.6	5.4	5.3	5.4	5.3	5.1	5.7
Bifidobacterium animalis										
0	6.4	6.4	6.4	6.4	6.4	6.4	6.4	6.4	6.4	6.4
2	5.0	5.2	5.3	5.2	5.3	5.1	5.2	5.3	5.2	5.5
4	5.1	4.7	4.8	5.2	5.1	4.8	5.0	5.3	5.1	5.3
6	5.3	4.3	4.7	5.1	5.5	5.0	5.0	5.5	5.3	5.2
8	5.2	4.5	4.6	5.1	5.0	5.5	5.2	5.0	5.1	5.1
10	4.2	4.6	4.7	4.4	4.8	4.6	4.4	4.1	4.0	5.5
Average	5.2	5.0	5.1	5.2	5.4	5.2	5.2	5.3	5.2	5.5
Lactobacillus paracasei subsp. *paracasei*										
0	6.4	6.4	6.4	6.4	6.4	6.4	6.4	6.4	6.4	6.4
2	6.6	6.4	6.3	6.4	6.5	6.4	6.4	6.2	6.2	6.8
4	6.5	6.2	6.3	6.2	6.3	6.4	6.0	6.0	6.0	6.5
6	6.4	6.1	6.2	6.1	6.2	6.1	5.9	6.2	5.7	6.4
8	6.4	6.1	6.2	6.1	6.0	6.1	5.8	6.2	6.0	6.4
10	6.4	6.1	6.0	5.7	5.9	6.9	5.5	5.5	6.7	6.2
Average	6.4	6.2	6.3	6.2	6.2	6.4	6.0	6.1	6.2	6.4
Propionibacterium freudenreichii subsp. *shermanii*										
0	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5
2	5.4	5.3	5.5	5.5	5.8	5.5	5.8	6.0	5.0	5.8
4	7.0	6.8	7.0	7.1	7.0	7.1	7.1	7.1	7.0	6.9
6	7.1	7.0	7.0	7.1	7.0	7.1	7.1	7.1	7.2	7.0
8	7.1	7.1	7.1	7.2	7.2	7.1	7.2	7.2	7.1	7.3
10	6.9	6.9	6.9	7.0	6.8	6.8	7.0	6.9	6.9	7.1
Average	6.8	6.7	6.8	6.9	6.9	6.9	6.9	7.0	6.8	6.9

was observed to reduce during the first few weeks and then stayed constant or started to increase slowly. The reduction was the highest (0.15 pH units) in treatments with the highest pH of 4.45, all other treatments showed a reduction of 0.08–0.10 pH unit. The control showed a reduction of 0.11 unit at the beginning and after that the pH stayed constant. The reduction in the pH during the first few weeks of the storage might be due to the metabolic activity and acid production by the probiotic bacteria. The higher reduction at pH 4.45 might indicate the highest metabolic activity of the organisms at this pH. The slight increase in pH might be due to the utilization and metabolism of the acids during the growth and proliferation by *P. freudenreichii* subsp. *shermanii*. The pH pattern again might be an indication of microbial metabolic activity during the first 2 days, where acids might have been produced to reduce the pH. The reduction of all probiotic bacterial population occurred during this period of high metabolic activity.

3.3. The effect of oil and gum on the survival of probiotic bacteria

As observed in previous experiments, in all three treatments (oil and gum, oil only and control with no oil and no gum) populations of *L. acidophilus* and *B. animalis* dropped rapidly during first 2 days and continued to drop slowly to result around 0.8 log unit for *L. acidophilus* and 1.3 log units for *B. animalis*. Other than this, no statistical significance between treatments could be observed. The population of *L. paracasei* subsp. *paracasei* was maintained at the inoculated rate throughout the shelf life. Although *P. freudenreichii* subsp. *shermanii* showed better growth in the presence

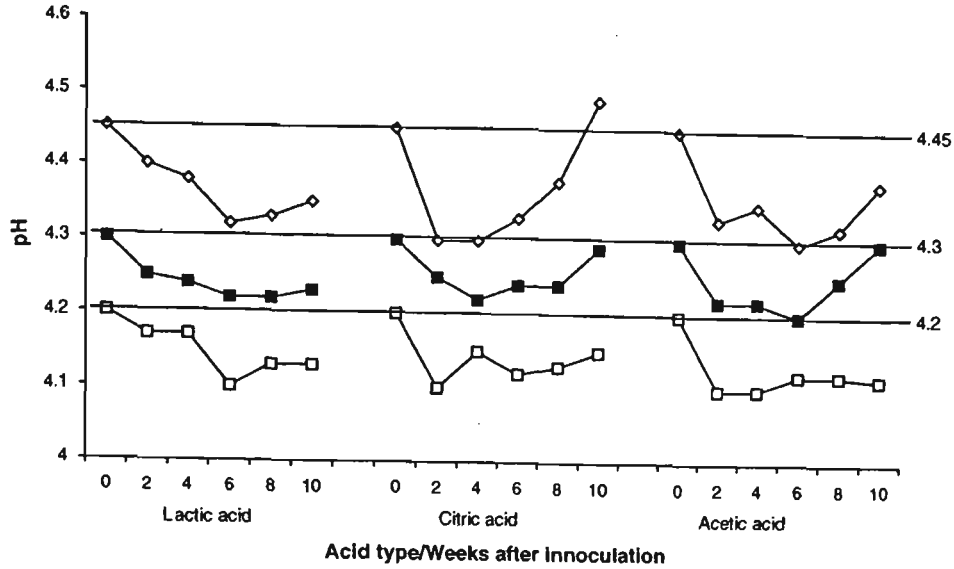

Fig. 1. Changes in the pH of dips made with different acids (lactic, acetic or citric acid) with different initial pH (4.45, 4.30, and 4.20), over a period of 10 weeks

of oil and gum, this effect was not statistically significant (data not shown).

3.4. *Effect of* L-cysteine · HCl *and* $NaHCO_3$ *on the survival of probiotic bacteria*

On average, the bacterial population (averaged over 10 weeks) was greater in the ABC (*L. acidophilus* (A), *B. animalis* (B) and *L. paracasei* subsp. *paracasei* (C) combination (7.2 log) than in the ABR (*L. acidophilus* (A), *B. animalis* (B) *L. rhamnosus* (R)) combination (6.8 log), though the inoculation rate was the same. In both the combinations, the population of *B. animalis* was greater than *L. paracasei* subsp. *paracasei*, *L. rhamnosus* and *L. acidophilus*. This might be due to the higher inoculation level (8 log cfu g^{-1}) of *B. animalis* as speculated in the earlier experiment. The populations of all the bacteria were greater in dips with $NaHCO_3$ than L-cysteine and control. The higher population in dips with $NaHCO_3$ may be due to reduced acid effect as a result of buffering. Though L-cysteine was expected to increase the population of probiotic bacteria by reducing redox potential, the effect was observed to be the opposite. Dave and Shah (1997) observed the same effect with bifidobacteria. The changes in bacterial population over time in both bacterial combinations and treatments are shown in Fig. 2. The population of *B. animalis* remained at similar levels in the control and with additives until week 2. After week 2, the population of *B. animalis* started to drop at a faster rate in dip with L-cysteine and at a relatively slower rate in dips with $NaHCO_3$. In both the bacterial combinations, the population of *L. acidophilus* dropped to 6 logs or below by week 2 and remained almost at that level till week 10. This might be due to the inhibition by *L. paracasei* subsp. *paracasei*, *L. rhamnosus* or *B. animalis*. However, in ABR combination the drop in *L. acidophilus* population was very drastic (<6 log) suggesting a greater inhibitory effect of *L. rhamnosus* on *L. acidophilus* than *L. paracasei* subsp. *paracasei*. The population of *L. paracasei* subsp. *paracasei* and *L. rhamnosus* was unaffected by additives or days in shelf.

4. Conclusion

This study has shown that French onion dip can be used as an effective carrier for probiotic bacteria when inoculated at 9 log g^{-1} or more. Bacterial combinations affected *L. acidophilus*, and *B. animalis*. When inoculated at 9 log g^{-1} or more, *L. acidophilus* and *B. animalis* population can be maintained above required level for health benefit over the storage period of 10 weeks. *L. paracasei* subsp. *paracasei*, *L. rhamnosus* and *P. freudenreichii* subsp. *shermanii* were not adversely affected by any of the bacteria in any combinations and can be inoculated at a rate of 7 log to maintain a population above 6 logs over the storage period of 10 weeks. The antagonistic effect with *L. paracasei* subsp. *paracasei* and *L. rhamnosus* needs further investigation. Selecting probiotic combination that show little or no antagonistic effect towards each other and the level of inoculation are the critical factors to maintain high population levels of probiotic bacteria in the dip. The combinations with *L. acidophilus*, *B. animalis*, *L. paracasei* subsp. *paracasei* and *P. freudenreichii* subsp. *shermanii* and *L. acidophilus*, *B. animalis*, *L. paracasei* subsp. *paracasei* and *P. freudenreichii* subsp. *shermanii* and *L. rhamnosus* can be used as probiotic combinations. However, the combination with *L. paracasei* subsp. *paracasei* was better than the combination with *L. rhamnosus* as a probiotic consortium.

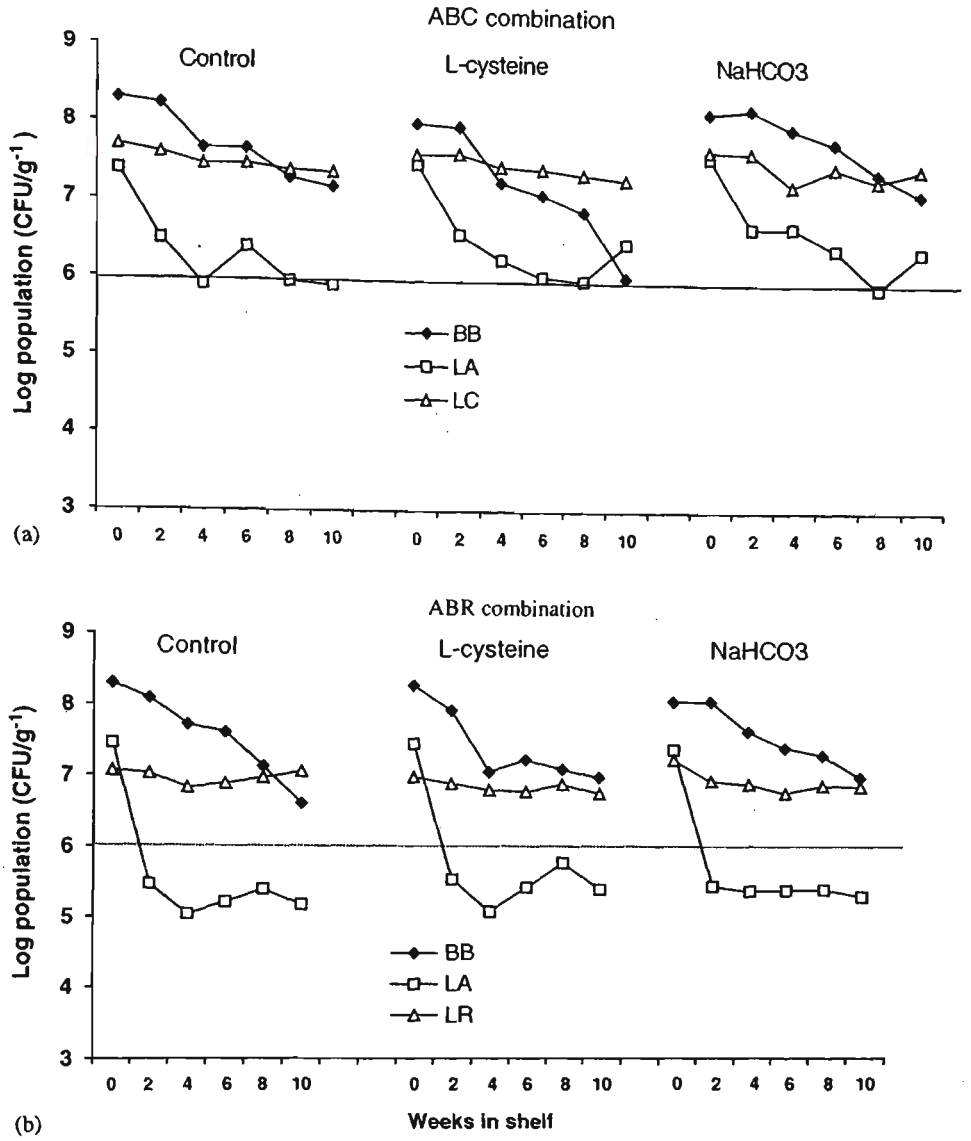

Fig. 2. Effect of addition of L-cysteine, $NaHCO_3$ to French onion dip and bacterial combinations either *Lactobacillus acidophilus*; LA, *Bifidobacterium animalis*; BB and *Lactobacillus casei*; LC (ABC; (a) or *Lactobacillus acidophilus*; LA, *Bifidobacterium animalis*; BB and *Lactobacillus casei* ssp. *rhamnosus*; LR (ABR; (b) on log population of LA, BB and LC or LR over a period of 10 weeks. 'Control' treatment did not have any chemicals.

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