PHYSICO-CHEMICAL AND THERAPEUTIC PROPERTIES OF LOW-FAT YOGURT AS INFLUENCED BY FAT REPLACERS, EXOPOLYSACCHARIDES AND PROBIOTICS

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DOCTOR OF PHILOSOPHY

by

Lata Ramchandran
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School of Biomedical and Health Sciences
Faculty of Health, Engineering and Science
Victoria University, Werribee Campus, VIC, Australia

Dedicated to the loving memory of my father

LATE SHRI T. K. RAMACHANDRAN

Abstract

The general objective of this research was to develop a low-fat probiotic yogurt using fat replacers and EPS producing strains of *Streptococcus thermophilus* along with probiotics and to establish its antihypertensive property *in vitro* and *in vivo*. The specific aims of this project were: a) to select suitable strains of *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* and of probiotics (*L. casei*, *L. acidophilus* and *Bifidobacterium longum*) based on their proteolytic, ACE-inhibitory and α-glucosidase inhibitory activities; b) to study the influence of a fibre-based fat replacer (Raftiline HP) and a protein-based fat replacer (Versagel) on the growth, proteolytic, ACE-inhibitory and α-glucosidase inhibitory activities of selected LAB; c) to select a suitable level of incorporation of the fat replacers in low-fat yogurt to obtain desired textural characteristics and to study the influence of these additions on the growth, proteolytic and ACE-inhibitory activities of *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* during storage; d) to study the protective role of EPS, with and without the selected fat replacer and/or probiotics on growth and survival of *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* and probiotics; yield of EPS, proteolytic and ACE-inhibitory activities; and spontaneous whey separation and textural properties of low-fat yogurt during storage; and e) to study the anti-hypertensive and hypocholesterolemic effects of the low-fat yogurt developed in this study by conducting a feeding trial using rats as a model.

The experiments were conducted in four phases. In the first phase, yogurt starters and probiotics were selected based mainly on their proteolytic and angiotensin-I-converting enzyme (ACE)-inhibitory properties. For this phase the growth, proteolytic enzyme profiles as well as proteolytic, ACE- and α-glucosidase (α-glu) inhibitory activities for two strains each of yogurt bacteria (*Streptococcus thermophilus* - 1275 and 285, and *Lactobacillus delbrueckii* ssp. *bulgaricus* - 1092 and 1368), and probiotic organisms (*Lactobacillus acidophilus* – 4461 and 33200, *Lactobacillus casei* – 2607 and 15286, and *Bifidobacterium longum* 5022) were examined by growing each organism individually in sterile reconstituted skim milk (RSM) at 37 °C for 6 h. Among all the strains of bacteria studied, both strains of *S. thermophilus* grew very well, while those of *L. delbrueckii* ssp. *bulgaricus* as well as the probiotics grew slowly. The growth pattern corresponded well with the decrease in pH for the organisms. All the organisms showed an increase in proteolysis with time. The variations in proteolytic capabilities translated into corresponding variations in ACE-inhibitory

activities of these organisms. *Bifidobacterium longum* 5022 showed highest ACE-inhibitory activity followed by *L. delbrueckii* ssp. *bulgaricus* 1368, *L. casei* 15286, *S. thermophilus* 1275 and *L. acidophilus* 4461. Organisms with high intracellular enzymatic activities grew well. Also, aminopeptidases of *L. acidophilus* 4461 and *S. thermophilus* 1275 that could better utilise proline containing substrates showed enhanced ACE-inhibitory activity. All organisms, particularly *L. casei* 2607, *L. acidophilus* 4461, *L. delbrueckii* ssp. *bulgaricus* 1092 and *B. longum* 5022 exhibited good α-glu inhibitory activity. Based on these results, the organisms selected for all further studies were: *S. thermophilus* 1275, *L. delbrueckii* ssp. *bulgaricus* 1368, *L. casei* 15286, *L. acidophilus* 4461 and *B. longum* 5022.

The second phase of the research involved the study of the effect of the addition of two fat replacers, Versagel® and Beneo HP® (earlier known as Raftiline HP®) on the growth and biochemical activities of the selected strains of bacteria. The influence of a protein-based fat replacer, Versagel added at 1 and 2% (w/v) to RSM, on the growth and metabolic activities of above selected strains were examined. Addition of Versagel improved growth of *S. thermophilus* 1275 and *B. longum* 5022 but inhibited that of *L. casei* 15286, *L. acidophilus* 4461 and *L. delbrueckii* ssp. *bulgaricus* 1368. This was also reflected in the extent of reduction of pH by these organisms in RSM with added Versagel. Addition of Versagel resulted in an increase in the quantity of lactic acid produced by *L. casei* 15286, *S. thermophilus* 1275 and *B. longum* 5022 but a decrease for *L. delbrueckii* ssp. *bulgaricus* in particular. Also, in the presence of Versagel, the organisms produced slightly higher quantities of acetic acid, particularly in RSM containing 2% Versagel. Among the biochemical activities, proteolytic activity of all the organisms except that of *B. longum* 5022, was adversely affected by the presence of Versagel, although the ACE-inhibitory and α-glu inhibitory activities were improved. Thus, Versagel at 1% level influenced the growth, while ACE-inhibitory and α-glu inhibitory activities of the organisms were better at 2% level.

The influence of a fibre-based fat replacer, Raftiline HP, added at the rate of 1, 2 and 3% (w/v) to RSM was examined on the growth and biochemical activities of selected strains of *S. thermophilus* 1275, *L. delbrueckii* ssp. *bulgaricus* 1368, *L. casei* 15286, *L. acidophilus* 4461, and *B. longum* 5022. The growth of *B. longum* 5022 and *S. thermophilus* 1275 was improved in RSM containing 1% Raftiline HP. All the organisms except for *S. thermophilus* 1275 produced more lactic acid and acetic acid in the presence of Raftiline HP than in the control. *Lactobacillus acidophilus* 4461 and *B. longum* 5022 showed improvement in the

proteolytic capabilities at all the three levels of Raftiline HP addition. *Lactobacillus delbrueckii* ssp. *bulgaricus* 1368 showed maximum percent ACE-inhibition in RSM containing 2% Raftiline HP while *B. longum* 5022 exhibited this potential in RSM containing 3% Raftiline HP. All organisms, except *L. delbrueckii* ssp. *bulgaricus* 1368, however, showed improvement in the α-glu inhibitory activity in RSM containing Raftiline HP.

The next step involved the selection of a suitable level of the fat replacers for the preparation of low-fat yogurt. The effect of Versagel on the growth and proteolytic activity of *S. thermophilus* 1275 and *L. delbrueckii* ssp. *bulgaricus* 1368 and ACE-inhibitory activity of the peptides generated thereby as well as on the physical properties of low-fat yogurt during a storage period of 28 days at 4 °C was studied. Three different types of low-fat yogurts, YV0 (control), YV1 (containing 1% added Versagel) and YV2 (containing 2% added Versagel), were prepared using Versagel as a fat replacer. The fermentation time of the low-fat yogurts containing Versagel was less than that of the control yogurt (YV0). The viable population of *S. thermophilus* 1275 was 8.68 to 8.81 log CFU/g and of *L. delbrueckii* ssp. *bulgaricus* 1368 was 8.51 to 8.81 log CFU/g in all the yogurts throughout the storage period. There was some decrease in the pH of the yogurts during storage and an increase in the concentration of lactic acid. However, the proteolytic and ACE-inhibitory potential of the starter cultures was suppressed in the presence of Versagel. On the other hand, the addition of Versagel had a positive impact on the physical properties of the low-fat yogurt, namely, spontaneous whey separation, firmness and pseudoplastic properties such as storage and loss moduli and flow behaviour.

The influence of incorporating Raftiline HP on the pH, growth, proteolytic and ACE-inhibitory activities and on spontaneous whey separation, firmness and rheological properties of low-fat yogurts during storage for 28 days at 4 °C was also investigated. Three types of yogurts were prepared from skim milk containing 0% (YI0, control), 2% (YI2) and 3% (YI3) Raftiline HP, respectively. The incorporation of Raftiline HP improved the growth of starter organisms, particularly that of *L. delbrueckii* ssp. *bulgaricus* 1368, resulting in shorter fermentation time. There was a significant improvement in total proteolysis which was highest in yogurt containing 3% Raftiline HP. The ACE-inhibitory activity was maximal in YI3 compared to YI2 and YI0. Incorporation of Raftiline HP did not affect whey separation and firmness of the low-fat yogurts. All these products were more fluid like with distinct pseudoplastic properties and lesser ability to resist deformation upon applied shear.

Based on the conclusions of these experiments, it was decided to use 3% Raftiline HP as a fat replacer in low-fat yogurt, which gave it the additional benefit as a prebiotic.

The third phase of the research studied the influence of using an exopolysaccharide (EPS) producing strain of *S. thermophilus* on the physico-chemical and physiological properties of low-fat yogurt. In this phase, the EPS producing strain of *S. thermophilus* – 1275 was used in the experimental batches of yogurt while a non-EPS producing strain of *S. thermophilus* – 1342 was used for making the control batches. First the influence of using EPS producing strain of *S. thermophilus* 1275 on the viability of yogurt starters, their proteolytic and ACE-inhibitory activities, as well on the textural and rheological properties of the low-fat yogurt during storage at 4 °C for 28 days was examined. The use of EPS producing strain of *S. thermophilus* 1275 did not have influence on pH, lactic acid content and the ACE-inhibition activity of low-fat yogurt. However, EPS showed a protective effect on the survival of *L. delbrueckii* ssp. *bulgaricus* 1368. Presence of EPS reduced the firmness, spontaneous whey separation, yield stress and hysteresis loop area but not the consistency and flow behaviour index of low-fat yogurt. The amount of EPS decreased during the storage of the yogurts.

The influence of using an EPS producing *S. thermophilus* 1275 along with 3% inulin (Beneo HP) on the viability of yogurt starters, their proteolytic, ACE- and α-glu-inhibitory activities, as well on the textural and rheological properties of low-fat yogurt during storage at 4 °C for 28 d was examined. The time to reach a pH of 4.5 was less in the presence of EPS producing *S. thermophilus* 1275. However, during storage, EPS and inulin together did not influence the pH and lactic acid, and the effect on ACE-inhibition activity varied with the period of storage. Presence of EPS showed a protective effect on the survival of *L. delbrueckii* ssp. *bulgaricus* 1368 and partially on the extent of proteolysis. The α-glucosidase-inhibitory activity was more apparent in EPS containing yogurt. The yield of EPS varied with the period of storage, being maximal (110.77 mg/100g) at day 14. EPS containing yogurts showed lower firmness, spontaneous whey separation, storage modulus, yield stress, consistency index and hysteresis area than non-EPS yogurts. It was concluded that low-fat yogurt with a stable and compact texture having antihypertensive and antidiabetic potential could be obtained using EPS producing strain of *S. thermophilus* 1275 and inulin.

Finally, the influence of EPS produced *in situ* along with 3% inulin and probiotics on the viability of the organisms, their proteolytic and ACE-inhibitory activities, as well on the textural and rheological properties of low-fat probiotic yogurt during storage at 4 °C for 28

days was examined. Two types of yogurt were prepared using strains of *S. thermophilus* not producing EPS (1342, NEPY) and producing EPS (1275, EPY). The yield of crude EPS increased (by 2.4 times) until d 21 of storage. Presence of EPS showed a protective effect on the survival of *L. delbrueckii* ssp. *bulgaricus* 1368 and *L. acidophilus* 4461 but not on *S. thermophilus* 1275, *L. casei* 15286 and *B. longum* 5022. No changes in post-acidification, lactic acid content or the ACE-inhibition activity of the two types of yogurt were observed. Overall, EPS containing yogurts exhibited higher proteolysis in the presence of inulin and probiotics (0.698 units) than the corresponding control (0.563 units). The storage and loss moduli, yield stress, consistency index and thixotropic behaviour of both samples were similar at day 1 and the influence of EPS was observable only after day 7. Thus, it appears that the effect of EPS on the textural and rheological properties of low-fat yogurt was modified in the presence of probiotics.

Based on the results of the third phase of experimentation, the final phase of the research was conducted to confirm the ability of the low-fat yogurt to exhibit the antihypertensive property *in vivo*. To confirm such a benefit, the effects of yogurt- and probiotic yogurt-based diets on the weight gain, serum lipid profile and blood pressure (BP) were investigated in spontaneously hypertensive rats. Three dietary treatments were fed for 8 weeks: skim milk diet (Feed-C), skim milk diet supplemented with freeze dried low-fat yogurt (Feed-Y), and with freeze dried low-fat probiotic yogurt (Feed-PY). The total weight gain for the 8 week period was maximum in rats fed Feed-C (90 g) followed by those fed Feed-PY (85.7g) and Feed-Y (78.7g), indicating that the overall weight gains were lesser in the groups fed yogurt containing diets. The ACE-inhibitory activities of the experimental feeds were 26.5% (Feed-Y) and 36.0 % (Feed-PY) indicating that inclusion of probiotics could improve the ACE-inhibitory potential of the feeds. The control feed did not show any ACE-inhibitory activity. At the end of the feeding period the reduction in systolic BP of rats fed Feed-Y was 3.7% (-9.46 mm Hg) and 2.7% (-6.41 mm Hg) in those fed Feed-PY while reduction in diastolic BP was 30 (-9.41 mm Hg) and 44% (-13.84 mm Hg) respectively, in comparison to those fed Feed-C. The levels of serum triglycerides, total cholesterol and low density lipoprotein-cholesterol of rats fed the supplemented diets (98.45 and 88.22, 155.31 and 149.40, 118.16 and 95.98 mg/dL, respectively in group RY and RPY) were lower than those fed Feed-C (85.87, 205.42 and 141.31) while no changes in the levels of high density lipoprotein-cholesterol were observed. It was concluded that feeding diets supplemented with yogurts exhibited antihypertensive and hypocholesterolemic effects in spontaneously hypertensive rats.

Professor Nagendra P. Shah (M. Sc., Ph. D.)
Professor of Food Science and Technology
School of Biomedical and Health Sciences
Faculty of Health Engineering and Science
Victoria University, Werribee Campus
Victoria, Australia

Certificate

This is to certify that the thesis entitled "PHYSICO-CHEMICAL AND THERAPEUTIC PROPERTIES OF LOW-FAT YOGURT AS INFLUENCED BY FAT REPLACERS, EXOPOLYSACCHARIDES AND PROBIOTICS" submitted by Lata Ramchandran in partial fulfilment of the requirement for the award of the Doctor of Philosophy in Food Sciences at Victoria University is a record of the bonafide research work carried out by her under my personal guidance and supervision and the thesis has not previously formed the basis for the award of any degree, diploma or other similar title.

Werribee, Australia
(Professor N. P. Shah)
Thesis supervisor
Date:

Declaration

"I, Lata Ramchandran, declare that the Ph. D. thesis entitled PHYSICO-CHEMICAL AND THERAPEUTIC PROPERTIES OF LOW-FAT YOGURT AS INFLUENCED BY FAT REPLACERS, EXOPOLYSACCHARIDES AND PROBIOTICS is no more than 100,000 words in length including quotes and exclusive of tables, figures, appendices, bibliography, references and footnotes. This thesis contains no material that has been submitted previously, in whole or in part, for the award of any other academic degree or diploma. Except where otherwise indicated, this thesis is my own work".

Lata Ramchandran Date:

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Werribee, Australia

Date:

Research Papers

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Oral Presentations

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Poster Presentations

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Award

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List of Abbreviations

µL = microlitre(s)

ACE = angiotensin-I converting enzyme

BP = blood pressure

CEP = cell-envelope protease

CFU = colony forming units

CVD = cardiovascular diseases

d or D = day(s)

dL = decilitre

DP = degree of polymerization

EMP = Embden-Meyerhof-Parnas

EPS = exopolysaccharide(s)

GIT = gastro-intestinal tract

G + C = guanine plus cytosine

h = hour(s)

HCl = hydrochloric acid

HDL = high-density lipoprotein

HPLC = high performance liquid chromatography

kJ = kilojoules

L = litre

LAB = lactic acid bacteria

LDL = low-density lipoprotein

M17 = selective medium for *S. thermophilus*

M = Molar

min = minute(s)

mL = millilitre

MPP = microparticulated protein

MRS = deMan Rogosa Sharpe

nm = nanometer

OPA = o-phthaldialdehyde

Pa = Pascal

RSM = reconstituted skim milk

s = second(s)

SHR = spontaneously hypertensive rats

ssp = subspecies

TCA = trichloroacetic acid

TG = triglyceride

UV = ultraviolet

v/v = volume/volume

w/v = weight/volume

WPC = whey protein concentrate

α-glu = α-glucosidase

1.0 Introduction

Increasing industrialization, urbanization and mechanization have led to dramatic changes in dietary pattern and lifestyle of the masses which in turn has caused an increase in the occurrence of chronic noncommunicable diseases such as obesity, diabetes mellitus, cardiovascular diseases, hypertension and stroke, and some types of cancer. Of these, the prevalence of overweight and obesity has been increasing at an alarming rate world over in the last two decades, being two-fold in USA and nearly 2.5-fold in Australia. Associations have been observed between obesity and type 2 diabetes, cardiovascular diseases and hypertension. Therefore, in recent years, people seeking a healthier life style prefer diets having low- or reduced-fat and fat free foods; this has led to the development of functional foods (Roberfroid, 1999a; WHO, 2002; Cameron et al., 2003; FAO/WHO, 2003).

Functional foods can be defined as foods 'that can beneficially affect one or more target functions in the body, beyond adequate nutritional effect, in a way relevant to an improved state of health and well being and/or reduction of risk of disease' (Stanton et al., 2005). Dairy products, particularly those containing probiotics, prebiotics and synbiotics are most popular in this category of foods. Probiosis can be defined as 'the positive effect of consumption of fermented dairy products with culture of lactic acid bacteria (LAB) on the equilibrium of intestinal microflora' (Tomasik and Tomasik, 2003). The benefits of consuming these organisms include maintenance of gut health, increased bioaccessibility of lipids and proteins, reduced allerginicity of foods and those resulting from the production of B-group and K vitamins, short chain fatty acids, polyamines, ω-3 unsaturated fatty acids including conjugated linoleic acid, and bioactive metabolites (Marteau et al., 1990; Tuohy et al., 2003; Stanton et al., 2005; Santos et al., 2006).

Prebiotics are defined as 'nondigestible substances that exert some biological effect on humans by selective stimulation of growth or bioactivity of beneficial microorganisms either present or therapeutically introduced into the intestine' (Roberfroid, 1998). Prebiotics of proven efficacy include fructooligosaccharides (FOS), inulin, lactulose and galacto-oligosaccharides. These oligosaccharides have the ability to beneficially alter the gut microbiota and also have the potential to reduce the risk of colorectal cancer, stimulate immune response, alleviate symptoms of inflammatory bowel disease, modify serum triglycerides and cholesterol, enhance mineral absorption in the intestine and thereby reduce the risk of intestinal infectious diseases, cardiovascular disease, non-insulin dependent diabetes, obesity, osteoporosis and cancer (Roberfroid, 2000; Williams and Jackson, 2002; Tuohy et al., 2003; Shah, 2007).

Synbiotics can be defined as ‘a mixture of probiotics and prebiotics that beneficially affects the host by improving the survival and implantation of live microbial dietary supplements in the gastrointestinal tract, by selectively stimulating the growth and/or by activating the metabolism of one or a limited number of health promoting bacteria, and thus improving host welfare’ (Tuohy et al., 2003). Experiments have provided evidence that synbiotics perform better than either probiotics or prebiotics alone in affecting the blood lipid profile and protecting from colorectal cancer (Gallaher and Gill, 1999; Tuohy et al., 2003) but require further investigations.

Since most of the probiotics are sensitive to the food environment, such as acidity and dissolved oxygen, short shelf-life products like yogurt are the most common functional foods in market (Shah, 2003; Stanton et al., 2003a). However, the health conscious consumer now demands more health benefits from this product, which has opened up new areas for research. The demand for low-fat yogurt with added benefits of pro- and pre-biotics is on the rise. Thus, it would be interesting to study the antihypertensive effect, both *in vitro* and *in vivo*, of low-fat yogurt containing fat replacers and probiotics.

According to FAO/WHO standards, yogurt is ‘the coagulated milk product obtained by lactic acid fermentation through the action of *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus*’ (Krasaekoopt et al., 2005). However, in recent years yogurts containing probiotics have gained popularity. These products contain *Lactobacillus* and *Bifidobacterium* species at 10^6 viable cells per millilitre of the product at the time of consumption (Arunachalam, 1999). Although there is no daily-recommended dose of prebiotics, doses of 4-20 g/d is required to show health benefits (Tuohy et al., 2003).

The most important textural characteristics of yogurt, firmness and ability to retain water, which results in a smooth viscous gel, are a major concern to manufacturers of low-fat yogurt. These characteristics, related to the gel structure, contribute to a smooth mouth feel of the product. Exopolysaccharides (EPS) produced by LAB, which are generally recognized as safe, are widely used to improve the body and texture of yogurt (Faber et al., 2001; Broadbent et al., 2003). Yogurt made with EPS producing cultures has better water binding capacity, which decreases the product’s susceptibility to syneresis (Hassan et al., 1995, 1996a; Amatayakul et al., 2006b). However, no simple correlation has been established between viscosity and quantity of EPS produced. It is opined that it may be more beneficial to use a combination of ropy and non-ropy starter cultures than using only ropy strains (Laws and Marshall, 2001). Moreover, the amount and type of EPS produced in milk by different species varies considerably and is also influenced by the growth conditions. Thus,

the functionality of EPS starters depends on the nature of polysaccharide, its composition, structure, type of linkage, branching and side groups as well as its interaction with other constituents of milk (De Vuyst et al., 2003; Ruas-Madiedo et al., 2005; Lin and Chang Chien, 2007). However, the interaction of EPS with other milk components has not been studied as yet to any great detail nor is the influence of EPS on the rheological and ACE-inhibitory properties of yogurts containing probiotics and fat replacers investigated. Therefore, it would be interesting to compare the rheological and physiological properties of EPS and non-EPS containing low-fat yogurts in the presence of the selected fat replacers and probiotics.

Fat replacers are also used to overcome the textural defects in low-fat/no-fat products. A fat replacer is an ingredient that can be used to provide some or all of the functions of fat, yielding fewer calories than fat. The major categories of fat replacers include carbohydrate based - (Avicel, Opta, Raftiline), protein based - (Simplesse, Dairy-Lo, Versagel) and fat based - (SALATRIM, OLESTRA) replacers. Among these, inulin has gained in popularity as it provides physiological benefits and lower caloric value (Jenkins et al., 1999; Roberfroid, 1999b) and can thus be used in foods designed for weight management.

During fermentation, LAB produce a range of secondary metabolites, some of which have been associated with health promoting properties of which the notable ones are the B vitamins and bioactive peptides. It is now well established that physiologically active peptides are produced from several food proteins during gastro-intestinal digestion and fermentation of food by LAB. ‘Peptides produced *in vivo* or *in vitro* by enzymatic hydrolysis of food proteins with biological functions or physiological effects are called bioactive peptides’ (Smacchi and Gobbetti, 2000). Digestive enzymes, naturally occurring milk enzymes, coagulants and microbial enzymes, especially from adventitious or starter LAB, generate bioactive peptides during milk fermentation and cheese maturation, thereby enriching the dairy products (Gobbetti et al., 2004a; Korhonen and Pihlanto, 2006). Upon oral administration, bioactive peptides, may affect the major body systems, namely, cardiovascular, nervous, gastrointestinal and immune systems, depending on the inherent amino acid composition and sequence. Among these, peptides with blood pressure lowering effects have received special attention and considerable significance is being attached to the role of diet in the prevention and treatment of the disease (López-Fandiño et al., 2006). Blood pressure regulation is partially dependent on the renin-angiotensin system (Silva and Malcata, 2005) in which the angiotensin-I converting enzyme (ACE) regulates the peripheral

blood pressure and hence its inhibition can exert an anti-hypertensive effect (Gobbetti et al., 2004a). However, it is now being suggested that these peptides may exert the physiological effect by other mechanisms also.

The production of ACE-inhibitory peptides *in situ* in dairy products is the most appealing approach of generating these peptides and the most effective way to increase the number of these peptides is to ferment or co-ferment with highly proteolytic strains of LAB. The challenge to this approach, however, lies in the selection of the right strain or a combination of strains (Meisel et al., 1997). ACE-inhibitory peptides produced in fermented milks using strains of proteolytic LAB (Nakamura et al., 1995a, 1995b; Seppo et al., 2002, 2003; Donkor et al., 2007b) and the proteolytic system of LAB are also well studied (Yamamoto et al., 1994, 1999; Juillard et al., 1995; Savijoki et al., 2006). Most of the ACE-inhibitory peptides originate from α_{s1}-, α_{s2}- and β- casein and β-lactoglobulin fractions of bovine milk and only a few among the large numbers identified as anti-hypertensive under *in vitro* conditions have proven to be clinically effective in animal and human studies (Korhonen and Pihlanto, 2006). However, there are no reports on the effect of fat replacers, prebiotics or probiotics on the ACE-inhibitory properties of products. It is not known whether the inclusion of such additives will have any influence, positive or otherwise, on therapeutic properties claimed in such products, such as antihypertensive properties. Therefore, to fill such a gap in knowledge, this forms the main focus of this research.

In this study, low-fat yogurt was formulated using fat replacers, EPS producing strain of *S. thermophilus* and probiotics and the influence of these on the physico-chemical, rheological and physiological properties were studied. Also, selection of strains of probiotics and *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* based on their proteolytic and ACE-inhibitory capabilities along with fat replacers, Versagel® and Raftiline HP®, which is also a prebiotic, formed the basis of the research and the best combinations were finally considered for *in vivo* testings.

The specific aims of this project were:

1. To select suitable strains of *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* and of probiotics (*L. casei*, *L. acidophilus* and *Bifidobacterium longum*) based on their proteolytic, ACE-inhibitory and α-glucosidase inhibitory activities;
2. To study the influence of a fibre-based fat replacer (Raftiline HP) and a protein-based fat replacer (Versagel) on the growth, proteolytic, ACE-inhibitory and α-glucosidase inhibitory activities of selected LAB;

3. To select a suitable level of incorporation of the fat replacers in low-fat yogurt to obtain desired textural characteristics and to study the influence of these additions on the growth, proteolytic and ACE-inhibitory activities of *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* during storage at 4 °C for 28 d;
4. To study the protective role of EPS, with and without the selected fat replacer and/or probiotics on growth and survival of *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* and probiotics; yield of EPS, proteolytic and ACE-inhibitory activities; and spontaneous whey separation and textural properties of low-fat yogurt during storage at 4 °C for 28 d; and
5. To study the anti-hypertensive and hypocholesterolemic effects of the low-fat yogurt that will be developed in this study by conducting a feeding trial using rats as a model.

A review of the relevant literature forms Chapter 2.0 of this thesis. Chapter 3.0 examines the growth, proteolytic and ACE- and α-glucosidase inhibitory activities of selected strains of bacteria grown in RSM, which was used as the selection criteria for organisms used in subsequent experiments. The influence of added Versagel and Beneo HP® (earlier known as Raftiline HP) on the growth, proteolytic and ACE- and α-glucosidase inhibitory activities of selected strains of the bacteria grown in RSM containing the fat replacers is described in Chapters 4.0 and 5.0, respectively. The effect of varying levels of the two fat replacers on the growth and survival of the yogurt starters, their proteolytic and ACE-inhibitory properties as well as whey separation, textural and rheological characteristics of low-fat yogurts is discussed in Chapters 6.0 and 7.0. Chapters 8.0, 9.0 and 10.0 investigate the influence of using an EPS producing strain of *S. thermophilus*, without inulin (Beneo HP) and probiotics; with inulin but without probiotics and with inulin and probiotics, respectively, on the survival of organisms, their proteolytic and ACE-inhibitory activities, yield of EPS as well as on the spontaneous whey separation, textural and rheological properties of the low-fat yogurts during storage at 4 °C for 28 d. The *in vivo* testing of the developed low-fat yogurt on rats, with and without probiotics, with regards to their antihypertensive and hypocholesterolemic effects is discussed in Chapter 11.0. The overall conclusions of this project are summarized in Chapter 12.0 and Chapter 13.0 focuses on the future directions of research. All relevant references are compiled in Chapter 14.0.

2.0 Review of Literature

2.1 Functional Foods

Over the last two decades, the changing concepts in nutrition have led to the introduction of functional foods. As the science of nutrition progresses, a wide variety of foods is being characterized as functional food with a range of components affecting a myriad of body functions relevant to either a state of well-being and health and/or to the reduction of the risk of a disease. Consequently, the term functional food has as many definitions as the number of authors referring to it (Roberfroid, 2005).

Based on some commonly used definitions, the term functional food can be broadly defined as ‘food and drink products derived from naturally occurring substances or those similar in appearance to conventional food or that which encompasses potentially helpful products including any modified food or food ingredient, that can and should be consumed as part of the daily diet and has been demonstrated to possess particular physiological benefits when ingested and/or reduce the risk of chronic disease beyond nutritional functions’ (Roberfroid, 1999a). Food Standards Australia and New Zealand, Australia's primary food regulatory agency, describes functional foods as ‘...similar in appearance to conventional foods and intended to be consumed as part of a normal diet, but modified to serve physiological roles beyond the provision of simple nutrient requirements’ (Food Standards Australia and New Zealand, 2006).

Industrialized nations as well as developing nations are facing several health related challenges. At the same time the modern concept of nutrition supports the hypothesis that, beyond providing nutrition, food can modulate various functions in the body that are relevant to health, thus emphasising on the promising use of foods to promote a state of well-being, better health and reduction of the risk of disease. These concepts are increasingly becoming popular with consumers. Advances in food science and technology have placed the food industry in the challenging position of addressing the growing consumer awareness of healthy foods.

The global functional food market has emerged from being a niche market to a mainstream market category and continues to be a dynamic and growing segment of the food industry. In 2004, the global functional foods market was estimated to be US$ 7.63 billion and expected to grow to US$167 billion by 2010 by which time the market is expected to mature. By 2010, the market size is expected to comprise approximately 5% of total food expenditures in the developed world (Anon., 2004). This growth is driven by consumers who

are seeking products that offer a solution to both long and short-term health problems. However, the Australian market for functional foods is in its infancy and is currently estimated at A$57.0 million with probiotic yogurt being the leader in this segment growing at 22%, and soy yogurt a strong second; together they dominate this market segment (Anon., 2007).

A food can be said to be functional if it meets one of the following criteria:

a. it contains a food component (being nutrient or not) which affects one or a limited number of function(s) in the body in a targeted way so as to have positive effects;
b. it has physiological or psychological effect beyond the traditional nutritional effect.

Collectively, a functional food should have a relevant effect on well-being and health or result in a reduction in disease risk. The component that makes the food "functional" can be 'either an essential macronutrient if it has specific physiological effects or an essential micronutrient if its intake is over and above the daily recommendations. Additionally, it could be a food component even though some of its nutritive value is not listed as essential, such as some oligosaccharides, or it is of non-nutritive value, such as live microorganisms or plant chemicals' (Roberfroid, 1999a).

The variety of functional foods that can be developed is driven by the imagination of scientists, the perceived benefits, and the willingness of consumers to pay for those benefits. The major types of functional foods are indicated in Table 2.1.

Table 2.1 Different types of functional foods.

Type	Description	Some examples
Fortified products	Increasing the content of existing nutrients	Grain products fortified with folic acid, fruit juices fortified with additional vitamin C
Enriched products	Adding new nutrients or components not normally found in a particular food	Fruit juices enriched with calcium, foods with probiotics and prebiotics
Altered products	Replace existing components with beneficial components	Low-fat foods with fat replacers
Enhanced commodities	Changes in the raw commodities that have altered nutrient composition	High lysine corn, carotenoid containing potatoes, lycopene enhanced tomatoes

(Source: Spence, 2006)

2.2 Functional Dairy Products

Dairy products are well-known as healthy natural products. Milk and dairy products constitute one of the four major food groups that make up a balanced diet. Apart from the nutritional benefits, milk has a potential role to play in the prevention of disease (Table 2.2). Dairy products are significant players in the functional food market accounting for approximately 60% of functional food sales in Europe (Shortt et al., 2004). In the US, they are the second most popular category of functional foods, with consumers spending $5.0 billion on functional dairy foods in 2004 (Vierhile, 2006).

Table 2.2 Dairy components and ingredients in functional foods and their health claims.

Ingredient	Sources	Claim areas
Minerals	Calcium Casein peptides	Optimum growth and development, dental health, osteoporosis
Fatty acids	CLA	Heart disease, cancer prevention, weight control
Prebiotics/carbohydrates	Galactooligosaccharides Lactulose Lactose	Digestion, pathogen prevention, gut flora balance, immunity, lactose intolerance
Probiotics	Lactic acid bacteria Bifidobacteria	Digestion, immunity, vitamin production, heart disease, antitumor activity, remission of inflammatory bowel disease, prevention of allergy, alleviation of diarrhea
Proteins/peptides	Caseins, whey proteins, immunoglobulins, lactoferrin, glycoproteins, specific peptides	Immunomodulation, growth, antibacterial activity, dental health, hypertension regulation (angiotensin inhibitors)

(Source: Shortt et al., 2004)

The most common functional dairy products are those with probiotic bacteria, quite frequently enriched with prebiotics, such as yogurt (Saxelin et al., 2003). Market analyst Datamonitor have evaluated the yogurt market in the United States to be about $7 billion and this is set to grow further. The increasing demand from consumers for dairy products with functional properties has been a key factor driving sales growth. This has led to the promotion of value-added products such as probiotic and other functional yogurts. By 2010,

the sales worldwide in probiotic yogurt category alone is expected to increase to $US500 million ($A587.3 million) from $US294 million ($A345.33 million) in 2007 (http://www.smh.com.au).

2.2.1. Probiotics

It has long been considered that the primary function of the human gastrointestinal tract (GIT) is simply to digest and absorb nutrients and excrete waste end-products. However, it is now accepted that the GIT fulfils many other functions that are essential to our well-being. The concept that the gastrointestinal microflora may have a role in maintaining human health is exciting, but it is not new. At the turn of the last century, Metchnikoff hypothesised that health and longevity in ethnic populations was in part a consequence of the composition of their intestinal flora. Since then, there has been great progress in scientific knowledge in the field of microbiology and the processes and consequences of bacterial fermentation. In the hope of achieving health benefits in the host, attempts have been made to manipulate the enteric microflora in a beneficial way, of which one technique has been to administer live microorganisms, termed probiotics, to normal subjects on the basis of /accepted health benefits or to a wide variety of patient groups (McNaught and MacFie, 2001).

Probiotics are defined as ‘live microorganisms that when administered in adequate amounts confer a health benefit on the host’ (FAO/WHO, 2002). Probiotic foods are ‘food products that contain a living probiotic organism in adequate concentration, so that after their ingestion, the postulated effect is obtained, and is beyond that of usual nutrient suppliers’ (Saxelin et al., 2003).

Probiotics consist of either yeast, especially *Saccharomyces*, or bacteria, especially certain LAB. The genera of bacteria (and yeast) that are commonly used as probiotics are listed in Table 2.3.

Table 2.3 Probiotic bacteria and fungi

Lactobacillus	*Bifidobacterium*	Others	Fungi
L. acidophilus	*B. bifidum*	*Bacillus cereus*	*Saccharomyces*
L. rhamnosus (GG)	*B. longum*	*Bacillus clausii*	*cerevisiae*
L. gasseri	*B. breve*	*Bacillus oligonitrophilis*	*Saccharomyces*
L. casei	*B. infantis*	*Clostridium butyricum*	*boulardii*
L. paracasei	*B. lactis*	*Escherichia coli* Nissle	
L. reuteri	*B. acolescentis*	1917	
L. plantarum	*B. thermophilum*	*Propionibacterium*	
L. cellobiosis	*B. animalis*	*freudenreichii*	
L. curvatus		*Enterococcus faecium*	
L. fermentum		*Lactococcus lactis*	
L. salivarius			
L. johnsonii			
L. helveticus			
L. farciminis			

(Source: Gorbach, 2002; Penner et al., 2005)

Of these the main species believed to have probiotic characteristics are *L. acidophilus, Bifidobacterium* and *L. casei*. Probiotic bacteria with desirable properties and well-documented clinical effects include *L. johnsonii* La1, *L. rhamnosus* GG, *L. casei* Shirota, *L. acidophilus* NCFB 1478, *B. animalis* Bb12 and *L. reuteri* (Shah, 2006b).

2.2.1.1 Criteria for selection of probiotics

Probiotics are generally recognized as safe. The effects due to their consumption can be direct or indirect through modulation of the endogenous flora or of the immune system. The active ingredients of probiotics which are responsible for the biological effects are often unknown, except for some enzymes and cell wall components with immunomodulating properties. However, their inclusion in food products with the purpose of conferring desired health benefits to consumers will depend on their ability to survive the human digestive system. The fate of probiotics in the GIT and consequent effects differ between strains. Some of them have a high survival capacity in the small intestine, and sometimes large intestine, whilst others are rapidly destroyed when they pass through the GIT. Some strains have the ability to adhere to epithelial cell lines while others do not. Usually they do not colonize the intestinal mucosa for long periods of time, and are eliminated within few days after the subject stops ingesting them; however, a few subjects have been shown to be colonized for long periods by some strains (Marteau, 2001).

For organisms to achieve probiotic status, they must fulfill a number of criteria (Marteau, 2001; Gorbach, 2002; Vasiljevic and Shah, 2008), such as:

1. be isolated from the same species as its intended host
2. have a demonstrable beneficial effect on the human health
3. be non-pathogenic
4. exhibit good growth characteristics, phage resistance and genetic stability
5. be able to survive transit through the GIT; resistance to acid and bile
6. be able to attach to human epithelial cells
7. be able to colonize the human intestine
8. produce an antimicrobial substance
9. large number of viable bacteria must be able to survive during processing and prolonged periods of storage

2.2.1.2 Therapeutic role of probiotics

The efficacy of probiotics to exert a positive influence on host health or physiology was not confirmed for long, as evidence for their efficacy was low and information on the stability of the strains in the products and their survival in the GIT was often lacking. However, a pharmacological approach has now been used to assess the effects and it is now known that that the pharmacokinetics of probiotics varies between strains and that many are indeed effective. The most interesting studies are related to the immunomodulatory effects of probiotics with regards to treatment of allergic disease or inflammatory bowel diseases (IBD) (Marteau et al., 2002). In recent times the proven benefits of probiotics in treatment and prevention of several diseases of GIT have been reviewed extensively (Vasiljevic and Shah, 2008). Interesting results have been published regarding food allergies and atopic eczema in children. Prevention of several infections and post surgical infections has also been reported. Promising results are being reported in patients with IBD and irritable bowel syndrome (IBS). It has also been suggested that probiotics could help treatment against *Helicobacter pylori* infection, but further studies are needed. Further, the role of probiotics in the process of carcinogenesis, as immune modulators in autoimmune disorders and even in conditions of the liver and skin are also being pursued (Gorbach, 2002). Some relevant studies reported and analysed during the past decade are highlighted in this section of the review.

2.2.1.2.1 Probiotics for diarrhoea

a) Rotaviral diarrhoea: Rotaviral diarrhoea occurs mainly in infants aged 6 months to 2 years. Effect of various probiotics on rotaviral diarrhoea has been investigated by double-blind, placebo-controlled randomized studies. In a multicenter study performed in Europe (Guandalini et al., 2000), 291 neonatal patients aged 1-3 months admitted for diarrhoea were randomly divided into 2 groups, and 10^{10} CFU of *L. rhamnosus* GG strain or placebo was administered after treatment of dehydration 4-6 h after admission. The results indicated that the duration of diarrhoea was shortened in the *L. rhamnosus* GG group, compared to the placebo group. In another double-blind, placebo-controlled randomized study performed in patients aged 6-36 months (75% were infected with rotavirus), ingestion of *L. reuteri* SD 2222 strain (10^{10}-10^{11} CFU) for 5 d was found to shorten the duration of watery diarrhoea (Shornikova et al., 1997). Furthermore, in a study performed in 175 nursery school children aged 6-36 months in Thailand, the test group was divided into powdered milk group, *Bifidobacterium* Bb 12-supplemented powdered milk group, and *Bifidobacterium* Bb 12 and *S. thermophilus* supplemented powdered milk group; and the anti-rotavirus IgA antibody titre on saliva was measured as an index of rotaviral infection (Saavedra et al., 1994). No increase in the antibody titre was noted in most subjects in the group that ingested *Bifidobacterium* Bb 12 and the group that ingested *Bifidobacterium* Bb 12 and *S. thermophilus*. Similarly, Szymański et al. (2006) have considered strains of *L. rhamnosus* (573L/1-3) to be effective in the treatment of rotaviral diarrhoea in children. Teran et al. (2009) also conducted a randomized, single-blind, controlled trial among 75 Bolivian children, aged 28 days to 24 months, suffering from acute rotavirus diarrhoea. They concluded that feeding of a probiotic mixture (*L. acidophilus, L. rhamnosus, B. longum*, and *Saccharomyces boulardii*) along with the regular medication was effective in the management of the disease in these children and reduced their duration of hospitalization as well as duration of diarrhoea. It is likely, that in the near future probiotics may become an integral part of treatment for acute diarrhoea for young children being treated for acute gastroenteritis (Heinz, 2008).

Additionally, preventive administration of probiotics for rotaviral infection disease has also been investigated. In a double-blind, placebo-controlled randomized study performed with 220 patients aged 1-18 months, the incidence of rotaviral infection was lower in patients fed maternal milk than in patients fed artificial milk, but daily preventive administration of 10^{10} CFU *L. rhamnosus* GG during hospital stay did not decrease the incidence (Mastretta et al., 2002). Similarly, Szajewska et al. (2001) have reported that

preventive administration of *L. rhamnosus* GG reduced diarrhoeal symptoms, but had no obvious prevention of rotaviral infection.

b) Antibiotic associated diarrhoea: Antibiotics cause diarrhoea due to an imbalance of intestinal bacterial flora in 20% of patients treated. In some cases, C*lostridium difficile* grows in large numbers and produces toxins that cause colonic damage. Therefore, many researchers and clinicians are trying to prevent or cure intestinal infections caused by *Cl. difficile* or *E. coli* O157-H7 with probiotics. C*lostridium difficile* is a Gram-positive, spore-forming bacillus. With the increasing use of broad-spectrum antibiotics the incidence of *Cl. difficile* associated diarrhoea has risen over the past two decades, particularly among elderly patients with prolonged hospitalization. Probiotics *L. rhamnosus* GG and *Saccharomyces boulardii* have been reported to have the potential to prevent antibiotic associated diarrhoea (Surawicz, 2003). Probiotic lactobacilli and bifidobacteria have been shown to suppress growth and subsequent adhesion of *Cl. difficile* (Naaber and Mikelsaar, 2004; Trejo et al., 2006) and thereby can be effective in preventing and reducing the severity of the disease (Graul, et al., 2009). Evidence from animal models indicates that pre-treatment with *Saccharomyces boulardii* can indirectly inhibit *Cl. difficile* toxin (Castagliuolo et al., 1996, 1999). The largest randomized, controlled trial with *Cl. difficile* associated colitis demonstrated that *Saccharomyces boulardii* (2×10^{10} CFU/d) was able to prevent recurrence of the disease (McFarland et al., 1994). Wullt et al. (2003) reported a similar effect using *L. plantarum* 299v. A controlled trial of *L. rhamnosus* GG to prevent antibiotic-associated diarrhoea symptoms in asymptomatic patients receiving antibiotics against *H. pylori* treatment showed substantial reduction in diarrhoea as well as symptoms of bloating and taste disturbance (Armuzzi et al., 2001). Meta-analysis of the effect of probiotics on antibiotic-induced diarrhoea in nine double-blind placebo-controlled studies has been performed, and the results clarified the significance of the action of probiotics in preventing recurrent *Cl. difficile* associated diarrhoea (D'Souza et al., 2002). Cremonini et al. (2002) have also found that probiotic regimens (*L. rhamnosus* GG, *Saccharomyces boulardii*, and a mixture of *L. acidophilus* and *B. lactis*) prevented diarrhoea associated with anti-*H. pylori* therapy. Yet another study of *Cl. difficile* associated diarrhoea demonstrated a clear reduction in the incidence of the disease with a probiotic product (*L. acidophilus* and *B. bifidum*) (Parkes et al., 2009). A similar observation was made by Hickson et al. (2007) in their trial using a fermented milk containing *L. casei* DN 114 001. However, it is suggested that the efficacy of probiotics is strain specific (Parkes et al., 2009).

c) Traveller's diarrhoea: Traveller's diarrhoea (3 times or more a day) is a common health problem among travellers. It occurs in residents of developed countries after travelling to subtropical and tropical zones. Most of the cases (80-85%) are due to bacterial pathogens, the most common one being one of the seven types of diarrheagenic *E. coli*. One of the reasons tourists become susceptible to this is that travel can disrupt the normal defence mechanisms of the body against infections by disturbing the normally protective bacteria in the intestines. Under such circumstances, probiotics can prove to be a promising therapeutic strategy as they can inhibit pathogen attachment, enhance immune response and assist in re-establishing normal microflora. A meta-analysis of 940 screened studies conducted over a period of 1977 to 2005 indicated that several probiotics (*Saccharomyces boulardii* and a mixture of *L. acidophilus* and *B. bifidum*) had noteworthy efficacy in the prevention of traveller's diarrhoea (McFarland, 2007).

2.2.1.2.2 Probiotics for infectious diseases

a) *Helicobacter pylori* induced infectious disease: *Helicobacter pylori* are microaerophilic Gram-negative rods. Colonization of the gastric mucosa by *H. pylori* is the main cause of gastritis and ulcers, and is strongly associated with gastric lymphoma and cancer. Since various strains of LAB and bifidobacteria prevented infection in animal models, and inhibited proliferation and urease activity of *H. pylori in vitro*, inhibition of *H. pylori* infection and its recurrence has been investigated in humans. *Helicobacter pylori* positive patients (n = 120) were divided into three groups that received the following treatments: i) eradication of the bacteria with drugs (eradication control group), ii) eradication + live *L. acidophilus* (live bacteria treatment group) and iii) eradication + killed *L. acidophilus* (killed bacteria treatment group). The groups receiving bacteria treatment (live or killed) along with eradication treatment showed eradication-promoting effect. Host immune system and inhibition of adsorption to glycolipid receptors were considered to be involved in the mechanism for the effect of killed bacteria, though not clarified (Canducci et al., 2000). In another study, 53 *H. pylori* positive patients were divided into 2 groups, and fermented milk containing *L. johnsonii* La1 strain was administered twice a day for 2 weeks in one group, and placebo was administered to the other group with the same schedule. On endoscopy and biopsy of the gastric mucosa, the density of *H. pylori* at the antrum and corpus of the stomach was decreased in the *L. johnsonii* treatment group. There is no clear description with regard to improvement of clinical symptoms in this report (Felley et al., 2001). In

Japan, when *H. pylori*-positive healthy subjects ingested yogurt containing *L. gasseri* OLL2716 (10^9 CFU/d bacterial count, 8 weeks), the $\Delta^{13}C$ value was decreased in an urea breath test, and the serum pepsinogen I/II ratio was increased compared to before ingestion indicating eradication of *H. pylori* (Sakamoto et al., 2001). In another study it has been shown that addition of *L. casei*-supplemented milk product to a standard triple therapy regimen in children resulted in a higher *H. pylori* eradication rate (Sykora et al., 2005). It has been observed that some probiotics exert antagonistic properties against *H. pylori in vitro* (Coconnier et al., 1998). Several studies have also shown the efficacy of probiotic cultures or their supernatants in decreasing gastric colonization by *H. pylori* and/or urease activity of this organism *in vivo* (Cremonini et al., 2001; Felley et al., 2001; Sakamoto et al., 2001). Another suggested mechanism of action is the inhibition of binding of *H. pylori* to its receptors by some probiotics, such as some *L. reuteri* strains (Mukai et al., 2002). A recent meta-analysis of randomized-controlled trials has shown that fermented milk-based probiotic preparations improve the rate of eradication of *H. pylori* by about 5-15% (Sachdeva and Nagpal, 2009).

b) Intestinal infections: Irritable bowel syndrome is the most common functional gastrointestinal disorder that exhibits symptoms such as abdominal discomfort, bloating, flatulence and faecal urgency. Neonatal stress may be correlated with functional GIT disorders such as IBS later in life. At present there is no universally effective curative treatment for IBS. It is believed that maternal use of probiotics may have prophylactic benefits for neonates at high risk of developing IBS in later life (Barouei et al., 2009). Kim et al. (2003) examined the effects of VSL#3 probiotic formulation on the symptoms of patients with diarrhoea predominant IBS. They did not find any global symptom relief, but observed a decrease in abdominal bloating in patients receiving the probiotic formulation. However, O'Mahony et al. (2005) found that treatment with *L. salivarius* or *B. infantis* lowered the symptom scores of the patients compared to those receiving placebo treatment. Brenner et al. (2009) have found *B. infantis* 35624 to be a safe and effective for treatment of IBS.

Nectrotising enterocolitis is the commonest gastrointestinal emergencies in neonates and the commonest cause of death and morbidity in preterm infants after first few postnatal days. Findings that link the establishment and composition of intestinal microbiota with healthy immune maturation and development of the disease have provided the rationale for using probiotics in such neonates. A review of the results from clinical trials suggests that specific probiotics might be useful in reducing the risk of necrotising enterocolitis and

infectious diseases in infancy (Rautava, 2007). Meta-analysis of 12 randomized controlled trials estimated a lower risk of necrotising enterocolitis, reduced risk of death and shorter time to full feeds in probiotic groups than in the controls (Deshpande et al., 2007).

c) Urogenital infections: The importance of maintenance of the healthy condition of intravaginal bacterial flora has recently been recognized in the field of gynaecology. Considering that maintenance of the dominant bacteria in normal vaginal microflora, *Lactobacillus*, at a high level is important for normal delivery and prevention of bacterial vaginosis, studies of introduction of probiotics into the vagina have been performed. Weekly administration of 10^9 CFU *L. rhamnosus* GR-1 and *L. fermentum* B-54 as a vaginal suppository for 1 year, decreased the incidence of urinary tract infection (Reid and Bruce, 1995). In an animal study it has been shown that activation of local immunity by *L. casei* Shirota strain played an important role in its ability to prevent urinary tract infection (Asahara et al., 2001). It has also been reported that when 10^9-10^{10} CFU *L. rhamnosus* GR-1 and *L. fermentum* RC-14 were orally administered, these probiotics transferred from the rectum to the vagina, causing reduction in the number of the harmful bacteria in the vagina, *E. coli* and fungi (Reid et al., 2003).

d) Respiratory infections: In a systematic review, Vouloumanou et al. (2009) evaluated the clinical evidence regarding use of probiotics for prevention of respiratory tract infections and concluded that probiotics appear to have a beneficial effect on the severity and duration of symptoms of respiratory tract infections but do not appear to reduce the incidence of such infections. Hatakka et al. (2007) have reported that probiotic treatment (*L. rhamnosus* GG and LC705, *B. breve* 99 and *Propionibacterium fredenreichii* JS) did not reduce the occurrence or recurrence of acute otitis media but showed a reduction in the occurrence of recurrent respiratory infections. Studies have suggested that probiotics (*L. rhamnosus* GG and *B. lactis* Bb-12) could offer a safe means of reducing the risk of otitis media and recurring respiratory infection during the first year of life (Rautava et al., 2009).

e) Pancreatitis: Pancreatic necrosis and associated pancreatic infections are the outcomes of the nature of microbial species inhabiting the intestine. Two randomized double-blind trials conducted indicate that treatment with *L. plantarum* (live and killed) appreciably lowered infection rates in patients with acute pancreatitis (Olah et al., 2002; Kecskes et al., 2003).

2.2.1.2.3 Probiotics for allergic diseases

In the last few years many studies have shown that prevalence of allergic diseases in western countries has increased in comparison to developing countries. It is now proposed that allergic diseases result from a fundamental failure of underlying immune regulation (Prescott and Björkstén, 2007). One of the reasons attributed to such allergic diseases are the differences in the composition of gut microflora of infants with high and low prevalence of allergy (Sepp et al., 1997). Lactobacilli and bifidobacteria were more frequent in non-allergic, whereas coliforms and *Staphylococcus aureus* were more common in allergic children (Bjorksten et al., 1999). Population based studies suggest that increased exposure to bacteria in early life can be protective against allergy (Marteau et al., 2002).

Atopic dermatitis in children is believed to be the starting point for subsequent allergic diseases such as asthma and allergic rhinitis (Spergel and Paller, 2003). This progression of clinical signs of atopic disease is referred as atopic march. Alterations in intestinal permeability and bacterial flora found in allergic patients with atopic disease and respiratory disease suggest a functional link between the respiratory and the digestive mucosal systems in atopic diseases (Munoz-Lopez, 2004). The increased intestinal permeability in allergic patients helps the passage of food allergy-inducing protein antigens from the diet; the first step in atopic march. Assessment of various strains of *Bifidobacterium*, *Lactobacillus* and *Lactococcus* have shown that administration of probiotics can restore the normal intestinal permeability thereby improving intestinal processing of antigens ingested in the diet, reducing intestinal inflammation and IgE production by increasing the uptake of antigens by Peyer's patches (gut-associated lymphoid tissue), and potentiation of regulatory T cell cytokines, consequently reducing symptoms of atopic dermatitis (Ji, 2009). In clinical trials, probiotics appear to be useful for the treatment of various clinical conditions related to atopic march. In 27 infants who manifested atopic eczema during exclusive breast-feeding, a reduction in severity of eczema was shown when the diet was supplemented with extensively hydrolysed whey formulas and *B. lactis* Bb12 or *L. rhamnosus* GG (Isolauri et al., 2000). In infants with cow's milk allergy and atopic eczema, the addition of *L. rhamnosus* GG to an extensively hydrolysed whey formula showed considerable improvement of atopic eczema and better anti-inflammatory properties (Majamaa and Isolauri, 1999). Pessi et al. (2000) reported a noteworthy increase in the concentration of indicators of anti-inflammatory properties (IL-10) in the sera of nine children with atopic disease and cow milk allergy treated with *L. rhamnosus* GG. Additionally, *L. rhamnosus* GG was observed to prevent the occurrence of atopic eczema in

infants at high risk up to 4 years (Kalliomäki et al., 2001; Rautava al., 2002). In one of the studies, in which *L. rhamnosus* and *L. reuteri* were given in combination, 56% of the patients experienced improvement of eczema compared with 15% in the placebo group (Rosenfeldt et al., 2003). A recent meta-analysis has suggested that novel probiotic strains may have a role in the management of eczema (Boyle et al., 2009). Similarly, administration of *L. rhamnosus* GG (Pohjavuori et al., 2004) and *L. fermentum* (Prescott et al., 2005) was associated with improvement in atopic dermatitis. Probiotics have also been evaluated to be effective in primary prevention of atopic disease; the preventative efficacy being observed even after 10 and 20 years of at-birth colonization of non-enteropathogenic *E. coli* (Lodinova-Zadaikova et al., 2003). Abrahamsson et al. (2007) concluded that although a preventative effect of probiotics (*L. reuteri* ATCC 55730; 1×10^8 CFU/day) on infant eczema was not confirmed, the treated infants had less IgE-associated eczema at 2 years of age and therefore could have a reduced risk to develop respiratory allergic disease later on. However, Wickens et al. (2008) found that supplementation with *L. rhamnosus* but not *B. animalis* ssp. *lactis* substantially reduced the cumulative prevalence of eczema, but not atopy, by 2 years. Thus, use of probiotics at an early age may lead to prevention of development of atopic march (del Guidice et al., 2006).

Further, Wang et al. (2004) reported an improved quality of life when 80 children suffering from perennial allergic rhinitis were administered fermented milk with the addition of *L. paracasei*-33. Arthur et al. (2009) have indicated that a probiotic combination of *L. acidophilus* NCFM and *B. lactis* BI-04 could prevent pollen-induced allergic rhinitis in children. However, it is not clear which probiotics would be effective and whether they would help cure allergy at other times of life. For example, *L. rhamnosus* GG administration did not prevent birch pollen allergy or apple allergy in allergic young adults or teenagers (Helin et al., 2002).

2.2.2 Prebiotics

Prebiotics are ‘non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon’ (Angus et al., 2005). Prebiotic foods are food products that contain a prebiotic ingredient in adequate concentration, so that after their ingestion, the postulated effect is obtained, and is beyond that of usual nutrient suppliers. At the present time prebiotics essentially constitute non-digestible oligosaccharides which stimulate the growth of bifidobacteria. These oligosaccharides are not or incompletely absorbed in the small

intestine. The most important prebiotics are glucans, fructans and mannans. Among the fructans, inulin and oligofructoses are commonly used (Oliveira et al., 2009a). These bioactive ingredients also exert a protective effect on probiotics in such products (Lerayer, 2005; Özer et al., 2005). This ingredient is discussed in greater details later in the review (Section 2.5.1.2). The tolerance of non-digestible oligosaccharides is usually very good. It depends on the type of the oligosaccharide, the time of consumption (worse in the fasting state than after meal), and individual factors such as absorption capacities, motility patterns, colonic responses, intestinal sensitivity, and the presence of IBS (Marteau, 2001). Some human studies have been conducted to study the benefit of administering prebiotics, predominantly in patients suffering from ulcerative colitis. Lewis et al. (2005) found that FOS demonstrated antagonistic effects in humans with ulcerative colitis. A couple of studies have reported the benefits of using germinated barley foods as a source of prebiotics to reduce the severity of (Bamba et al., 2002) and prolong the duration of remission in ulcerative colitis (Kanauchi et al., 2003; Hanai et al., 2004). A recent review has indicated that prebiotics may show beneficial effects in human IBD and colitis and hence the emerging therapy for such diseases targets the function of intestinal microflora (Langen and Dieleman, 2009).

2.2.3 Synbiotics

Synbiotics are ‘mixtures of pro- and prebiotics that beneficially affect the host by improving the survival and implantation of selected live microbial strains in the gastrointestinal tract’ (Saxelin et al., 2003). The rationale behind synbiotic treatment is that the desired probiotic and prebiotic would together exert a greater beneficial effect than when administered individually. There are a few studies demonstrating the positive effects of synbiotic therapy, making it a more logical and viable treatment option. In a double-blinded randomized controlled trial, patients receiving a synbiotic (prebiotic Synergy and probiotic *B. longum*) demonstrated improvement in all clinical parameters of ulcerative colitis, such as improved sigmoidoscopy scores, decrease in human β-defensin mRNA and reduced inflammation in biopsies, thereby providing strong preliminary evidence that synbiotic therapy may be beneficial in the treatment of ulcerative colitis (Furrie et al., 2005). Further, In a preliminary investigation, Schouten et al. (2009) found that dietary intervention of mice with a synbiotic diet consisting of a prebiotic mixture (Immunofortis) and a probiotic (*B. breve* M-16V) reduced their allergic responses to cow milk allergens.

Prevention of infectious complications after surgery for digestive organs is a major clinical task. Taking into account that post-operative administration of antibiotics at a high dose for a long period promotes bacteria with a low sensitivity to antibiotics, the basic attitude in digestive organ surgery is to refrain from the use of antibiotics. Therefore, to prevent infectious diseases after such surgeries, probiotic and prebiotics have recently been introduced in this treatment regimen. The effect of enteral administrations of live and heat-inactivated *L. plantarum* 299v (supplemented with oat fibre) on development of sepsis after surgery in the abdominal cavity (hepatectomy, pancreatectomy, gastrectomy, resection of large intestine, and intestinal bypass) has been reported (Rayes et al., 2002). It was observed that this treatment appreciably decreased the incidence of sepsis compared to enteral nutrition, and was particularly effective for gastrectomy and pancreatectomy. Kanazawa et al. (2005) added probiotics (*L. casei* Shirota strain, *B. breve* Yakult strain, 5×10^9 CFU/g × 3g each) and prebiotics (galactooligosaccharides, 6.6g/day) to postoperative enteral nutrition for patients with infectious complications such as peritoneal bascess and sepsis, that occur at a high incidence after hepatectomy and extrahepatic bile duct resection and reconstruction of the biliary tract in patients with highly invasive biliary tract cancer, and investigated the protection from infections. This synbiotic therapy markedly improved intestinal microflora in the patients after surgery for bile duct cancer, and decreased the incidence of infectious complications. Furthermore, the quality of life of the patients was also improved, with shortening of the duration of post-operative hospital stay and the antibiotics administration period. Randomized controlled trials have also shown that the preparation VSL#3 is effective in post-surgical prevention of pouchitis following colectomy (Mimura et al., 2004).

2.3 Yogurt as a Functional Food

Fermented dairy products, having the tradition as healthy foods, are a natural choice for their makeover as functional foods. The word ‘yogurt’ was derived from the Turkish word ‘Jugurt’. A vast array of yogurts is now available in the market to suit all palates and meal occasions. Yogurts are available in a variety of textures (e.g. liquid, set, smooth), fat contents (luxury, low-fat, virtually fat-free) and flavours (natural, fruit, cereal). The low-fat varieties of yogurt provide an array of important nutrients in significant amounts in relation to their energy and fat content, therefore making them a nutrient-dense food (Shah, 2003; McKinley, 2005). The healthy image of yogurt is further endorsed by the addition of various

fruit preparations in yogurt to include the health benefits of fruits such as providing fibre and antioxidants (O'Rell and Chandan, 2006). In recent years soymilk (Donkor et al., 2007c; Champagne et al., 2009; Cruz et al., 2009; Ferragut et al., 2009), corn milk (Supavititpatana et al., 2008) and peanut milk (Isanga and Zhang, 2009) yogurts are being developed as a vegetarian alternate to bovine milk yogurt that can also overcome the problem of milk protein allergenicity. Further, inclusion of plant extracts to enhance yogurt functionality, such as tea catechins for antioxidative and antimicrobial properties (Jaziri et al., 2009), is also being considered.

Yogurt is defined as 'a product resulting from milk by fermentation with a mixed starter culture consisting of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*'. However, in some countries, including Australia, other suitable LAB are permitted for use as starter cultures. As a result, some yogurt manufacturers use *L. helveticus* and *L. jugurti* for yogurt manufacture. However, US standards do not permit the use of any starter culture other than *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* (Shah, 2003).

The Australian standards define low-fat yogurt as 'the yogurt prepared by culturing skim or low fat cows milk, resulting in a thickened, tangy yogurt and does not contain fruit or flavouring. It contains on an average 6.6% protein and 0.3% fat' (Food Standards Australia and New Zealand, 2006).

A starter culture can be defined as 'a microbial preparation of large number of cells of at least one microorganism to be added to a raw material to produce a fermented food by accelerating and steering its fermentation process' (Leroy and De Vuyst, 2004). During fermentation, lactic acid is produced from lactose by the yogurt bacteria, *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus*. These LAB also produce acetic acid, ethanol, aroma compounds, bacteriocins, exopolysaccharides, and several enzymes. In this way they enhance shelf life and microbial safety, improve texture and contribute to the pleasant sensory profile of the yogurt (Leroy and De Vuyst, 2004). The functional properties of LAB that contribute to the functionality of fermented products such as yogurt are listed in Table 2.4. Of the various functionalities, this review focuses on the health benefits that accrue from yogurts as a consequence of fermentation (Section 2.6.2).

Table 2.4 Contribution of LAB to functionality of fermented products.

Functional property	Contribution to food functionality
Production of exopolysaccharides, amylase, aroma generation	Safety and/or organoleptic
Bacteriophage resistance, prevention of overacidification in yogurt	Technological
Production of bioactives, nutraceuticals, reduction of toxic compounds and anti-nutritional compounds	Nutritional and health

(Compiled from Leroy and De Vuyst, 2004)

2.3.1 Characteristics of yogurt starters

a) *Streptococcus thermophilus*: The genus *Streptococcus* consists of Gram-positive, spherical-ovoid or coccobacillary cells. The guanine plus cytosine (G + C) content of the DNA of species of this genus is 34-46 mol%. The 39 currently classified species of the genus *Streptococcus sensu stricto* are grouped as: a) oral (*S. salivarius*, *S. mutans*, *S. mitis*, *S. thermophilus*); b) pyogenic (*S. pyogenes*, *S. agalactiae*) and c) other streptococci (*S. bovis*, *S. equinus* and *S. alactolyticus*) (Gobbetti and Corsetti, 2000). The only 'dairy streptococci' remaining from those originally described by Sherman more than 60 years ago is *S. thermophilus*.

Streptococcus thermophilus, like most LAB, is non-spore-forming, catalase-negative and facultatively anaerobic. These spherical or ovoid cells (0.7-0.9 µm diameter) occur in pairs or chains when grown in liquid media and in milk it occurs in long chains of 10-20 cells. It grows best at a temperature of about 42-45 °C. They are heterotrophic and generally fastidious, requiring simple carbohydrates as energy source, and preformed amino acids as a nitrogen source. It ferments lactose homofermentatively, to give L(+) lactic acid as the principal product. Lactose is actively transported across the cell membrane of *S. thermophilus*, by means of a membrane located enzyme, galactoside permease. Inside the cell, the enzyme β-galactosidase hydrolyses the lactose to glucose and galactose. The glucose is metabolized to pyruvate via the Embden-Meyerhof-Parnas (EMP) pathway, and lactic dehydrogenase converts the pyruvate to lactic acid. In most strains of *S. thermophilus*, the galactose and lactic acid produced leave the cell and accumulate in the medium, but some strains possess a galactokinase, that converts the galactose to galactose-1-phosphate which is converted via the Leloir pathway to glucose-1-phosphate, that is further metabolized via the EMP pathway (Robinson, 2000; Zirnstein and Hutkins, 2000).

b) *Lactobacillus delbrueckii* ssp. *bulgaricus*: The genus *Lactobacillus* is quite diverse and consists of a number of different species that have little in common. A measure of their diversity can be estimated by the range of G + C% content among the lactobacilli. Members of the species have G + C content of 32-53%, which is much wider than is encountered with other LAB. The lactobacilli include over 25 unique species, and the first level of differentiation is based on end product composition: homofermentators - classified as organisms that produce >85% lactic acid as their end product from glucose (e.g. *L. delbrueckii* ssp. *bulgaricus*, *L. acidophilus*), and heterofermentators - classified as organisms that produce approximately 50% lactic acid as the end product, with considerable amounts of carbon dioxide, acetate and ethanol (e.g. *L. brevis*, *L. casei*). Although they all produce lactic acid as a major end product they differ in the isomeric composition of lactic acid produced. Some produce exclusively L(+) lactic acid and these include *L. salivarus* and *L. casei*. Others, for example *L. delbrueckii* ssp. *bulgaricus* and *L. jensenii* produce just D(-) lactic acid, and finally *L. acidophilus* and *L. helveticus* produce a mixture of D(+) and L(-) lactic acid. Their optimum growth temperature is in the range of 30-40 °C. They are also aciduric with an optimum growth pH of 5.5-5.8 but in general they can grow at a pH of less than 5.0 (Batt, 2000).

Lactobacillus delbrueckii ssp. *bulgaricus* is also Gram-positive, but it occurs in milk as chains of 3 to 4 short rods, each 0.5-0.8 × 2.0-9.0 µm, with rounded ends. The optimum growth temperature is 45 °C. Its basic metabolism is homofermentative, to give D(-) lactic acid to a level of 1.7-2.1% in milk. It converts hexoses into lactic acid via the EMP pathway. Although lactic acid is the major end product of fermentation, secondary end products such as acetaldehyde, acetone, acetoin and diacetyl can also be produced in very low concentrations. *Lactobacillus delbrueckii* ssp. *bulgaricus* can, like *S. thermophilus*, utilize lactose, fructose and glucose, and some strains can utilize galactose (Robinson, 2000; Teixeira, 2000).

2.3.2 Probiotics in yogurt

Probiotic bacteria are adjuncts added to fermented milks such as yogurt. Owing to the health benefits, most bacteria with probiotic properties belong to the genera *Lactobacillus* and *Bifidobacterium*, which are common but non-dominant members of the indigenous microbiota of the human GIT. Some of the potential health benefits of functional foods containing probiotic bacteria include improved digestibility, improved nutritional value,

improved lactose utilization, antagonistic action towards enteric pathogens, colonization in gut, anticarcinogenic effect, hypocholesterolemic effect, immune modulation, prevention of allergy and prevention of inflammatory bowel disease (Gomes and Malcata, 1999; Vasiljevic and Shah, 2008). *Lactobacillus acidophilus, L. casei, L. paracasei* and *Bifidobacterium* species are predominantly used in yogurt (Holzapfel et al., 2001). Manufacturers of therapeutic fermented milk products commonly use five species of *Bifidobacterium* (*B. adolescentis, B. bifidum, B. breve, B. infantis* and *B. longum*) (Arunachalam, 1999).

The characteristics of probiotic strains vary, and each strain has to be studied individually. Some probiotic strains are sufficiently proteolytic to grow excellently in milk, but others need growth stimulants. Those that do not ferment lactose need monosaccharides. Sometimes the texture or the taste of a milk product fermented with a probiotic does not meet with consumer approval or is technologically impractical. For this reason it is common to use probiotic bacteria together with standard starter cultures as in yogurt (Saxelin et al., 2003). Most *Bifidobacterium* species cannot ferment milk by themselves because they require low redox potential and peptides generated from the breakdown of casein, a milk protein. Moreover, when co-cultured with lactobacilli, they become inhibited as the pH drops (Klaver et al., 1993). Several factors such as strain characteristics, food matrix, temperature, pH and accompanying microbes affect the viability of probiotics (Fondén, 2003). A synbiotic product containing the probiotic bacteria and prebiotic in a single food, can improve the survival of bifidobacteria during the storage of the product and during the passage to the intestinal tract, and also reduce the competition with microorganisms in the GIT (Lerayer, 2005).

The combined use of two or more probiotic species is common in commercial probiotic foods, as these strains are believed to act synergistically on each other. Thus, the trend is to use yogurt bacteria as the main starter culture and probiotic bacteria as an adjunct starter (Shah, 2006b). The most common probiotic dairy products worldwide are various types of yogurt, cultured buttermilks, various LAB drinks such as Yakult, and mixtures of probiotic fermented milks and fruit juice. A list of some probiotic yogurts and fermented milks commercially produced is presented in Table 2.5.

2.3.3 Characteristics of common probiotics

a) *Lactobacillus acidophilus*: This organism, first isolated by Moro in 1900 from infant faeces, has undergone many transformations in the description of its metabolic, taxonomic and functional characteristics. It is isolated from the intestinal tract of humans and animals

and is also reported in the faeces of milk-fed infants and older persons consuming high milk-, lactose- or dextrin-diets. These Gram-positive rods (0.5-1 × 2-10 µm), with rounded ends, occur in pairs or short chains. It is non-flagellated, non-motile and non-spore-forming, and is intolerant to salt. It was initially categorized in the thermobacteria classification of LAB based on their homofermentative metabolism and ability to grow at 45 °C. In 1980, *L. acidophilus* was recognized as a heterogenous group by DNA hybridization studies. This group, known as the *L. acidophilus* complex, is composed of the six distinct species of *L. acidophilus*, *L. crispatus*, *L. amylovorus*, *L. gallinarum*, *L. gasseri* and *L. johnsonii*. Although these are regarded as separate species, they are closely related and have been suggested as belonging to one phylogenetic group or branch. Cultures of *L. acidophilus* are microaerophilic and capable of aerobic growth in static cultures without shaking. They prefer anaerobic conditions and growth is stimulated in broth or agar under a standard anaerobic gas mixture of 5% carbon dioxide, 10% hydrogen and 85% nitrogen. The nutritional requirements of *L. acidophilus* reflect the fastidious nature of these bacteria. Currently, members of the *L. acidophilus* complex are classified as obligate homofermenters. Hexoses are fermented by this group primarily to lactic acid by EMP pathway. All species produce L and D isomers of lactic acid, the yield being 1.8 mol/mol glucose. Additionally, they produce a variety of antimicrobial compounds, including lactic acid, hydrogen peroxide and a variety of bacteriocins (Gomes and Malcata, 1999; Klaenhammer and Russell, 2000; Holzapfel et al., 2001).

b) *Lactobacillus casei*: This is a typical cheese bacterium isolated mainly from silage, sourdough, cow dung, human intestinal tract, mouth and vagina. It is a Gram-positive, non-motile, non-sporulating and catalase-negative bacterium having an optimum growth temperature of 30 °C. These cells are rods of 0.7-1.1 × 2.0-4.0 µm, often with square ends, which tend to form chains. Bergey's Manual of Systematic Bacteriology recognizes four subspecies: *casei*, *pseudoplantarum*, *rhamnosus* and *tolerans*. The latest grouping of lactobacilli based on chemical-physiological criteria includes *L. casei* in the facultatively heterofermentative group. Hexoses are almost entirely converted into lactic acid via EMP pathway and pentoses are used by induced phosphoketolase, to produce lactic acid and acetic acid (Gobbetti, 2000).

Table 2.5 Probiotic dairy products available in the market.

Product	Country of origin	Organism(s)
ACO-yogurt	Switzerland	*S. thermophilus, L. bulgaricus, L. acidophilus*
Cultura-AB	Denmark	*L. acidophilus, B. bifidum*
AB-yogurt	Denmark	*L. acidophilus, B. bifidum, S. thermophilus*
Biogarde	Germany	*L. acidophilus, B. bifidum, S. thermophilus*
Bifighurt	Germany	*B. longum, S. thermophilus*
Gefilac	Finland	*L. casei* GG (rhamnosus)
Yakult	Japan	*L. casei*
Miru Miru	Japan	*L. acidophilus, L. casei*
Biokys	Slovakia	*B. bifidum, L. acidophilus, Pediococcus acidilacto*
Ofilus	France	*B. bifidum, B. longum, L. acidophilus, S. lactis, S. cremoris*
Gaio	Denmark	*E. faecium, S. thermophilus*
LC1	Europe	*L. acidophilus* La1
Symbalance*	Switzerland	*L. reuteri, L. casei, L. acidophilus*
Probiotic plus oligofructose	Germany	*L. acidophilus, L. bifidus,* LA7
ProCult3	Germany	*B. longum* BB536
Actimel Orange	Germany	*L. acidophilus*
Fysiq*	Netherlands	*L. acidophilus* Gilliland
DanActive Immunity	USA	*L. casei Immunitas*™
Activia	USA	*B. animalis*
Morinaga BB536 Nomu Drinking Yogurt	Japan	*B. longum* BB536
Megumi series	Japan	*L. gasseri* SP, *Bifidobacterium* SP
Actimel (Danone)	France	*L. casei* Immunitass DN-114001
Yakult Light	Netherlands	*L. casei* Shirota
Vitality	UK	*Bifidobacterium* spp., *L. acidophilus*

* contains Fructo-oligosaccharide

(Compiled from Daly, 1991; Vierhile, 2006; Alhaj et al., 2007)

c) *Bifidobacterium*: Bifidobacteria were once largely unknown by people working in the area of food science and technology, but since the mid-1980s there has been a revival of interest due to the expanded use of bifidobacteria in products that are now marketed as functional foods. The G + C% of DNA for *Bifidobacterium* varies from 54 to 67%. Bergey's Manual of Systematic Bacteriology identifies 24 species of *Bifidobacterium*, of which the types considered primarily human in origin are the species: *bifidum*, *longum*, *infantis*, *breve*, *adolescentis*, *angulatum*, *catenulatum*, *pseudocatenulatum* and *dentium*. Organisms of the genus *Bifidobacterium* are short, regular, thin rods (0.5-1.3 × 1.5-8 μm) that are slightly bifurcated club-shaped elements in star-like aggregates or disposed in 'V' or 'palisade' arrangements. They are Gram-positive, usually catalase-negative, non-spore-forming, non-motile cells. They are anaerobic but some species are aero-tolerant. The optimum growth temperature is in the range of 37 to 41 °C and the optimum pH for initial growth is 6.5-7.0. They metabolize glucose exclusively by heterolactic fermentation by the fructose-6-phosphate shunt also know as bifid shunt, to form L(+) lactic acid and acetic acid in the molar ratio of 2:3 (Holt et al., 1994; Arunachalam, 1999; Gomes and Malcata, 1999; Hoover, 2000). Besides glucose, all bifidobacteria from human origin are also able to utilize galactose, lactose and fructose as carbon sources. A proton symport has been identified as the lactose transport system for *B. bifidum* DSM 20082 (Krzewinski et al., 1996). In some instances they are also able to ferment complex carbohydrates as reported by Crociani et al. (1994).

Some major advances in starter and non-starter LAB during the past decade has been reviewed by Cogan et al. (2007).

2.3.4 Manufacture of yogurt

The method of yogurt making has changed very little over the years. The major steps in yogurt making are outlined in Figure 2.1. Cow's milk is generally used as the raw material for yogurt production, although the milk from sheep, camel and buffalo is equally suitable for fermentation. However, the low level of α_{s1}-casein in goat's milk causes the formation of a softer coagulum and thereby results in a product that lacks the typical 'mouth feel' of yogurt. One of the critical features of the milk used for yogurt production is the level of solids-not-fat (SNF). In cow's milk, the SNF level is 8.5-9.0%. However, the levels of protein in the milk (3.3%, of which 2.6% is casein and 0.7% is whey proteins) is not sufficiently high to produce a satisfactory end product, and so the first step in manufacture is to raise SNF content to about 14-16% (Robinson, 2000).

The fermentation process involves the inoculation of pasteurized milk that has been enriched in milk protein with concentrated cultures of bacteria; the milk is then incubated at 40 to 44 °C for 4 to 5 h. During fermentation lactic acid is produced from lactose by the yogurt bacteria. The reduction in pH, due to the production of lactic acid, causes destabilization of the micellar casein at a pH of 5.1 to 5.2, with complete coagulation occurring around pH 4.6. At the desired final pH, the coagulated milk is cooled quickly to 4 to 10 °C to slow down the fermentation process. The metabolic activities of LAB are responsible for the production of lactic acid, the coagulation of milk proteins, and the production of various compounds that decide the organoleptic and textural characteristics of the final product (Water, 2003).

2.3.5 Problems in low-fat yogurt

Traditionally the solids content of milk is increased up to 18% for yogurt production. Increasing total solids by fortification with dairy ingredients increases the concentration of proteins by 4-5% and results in improved yogurt texture. However, high level of fortification with milk solids can lead to certain problems such as powdery taste in the yogurt and cause excessive acid development, especially during storage (Mistry and Hassan, 1992). Additionally, heat treatment of milk at temperatures above 70 °C prior to fermentation is common for increasing the gel firmness and reducing the level of syneresis. This effect is a consequence of whey protein denaturation during the heat treatment as these denatured proteins are more susceptible to inter-protein aggregation with other denatured whey proteins or with casein micelles. Some of the textural benefits that accrue from some of these commonly adapted steps are summarized in Table 2.6.

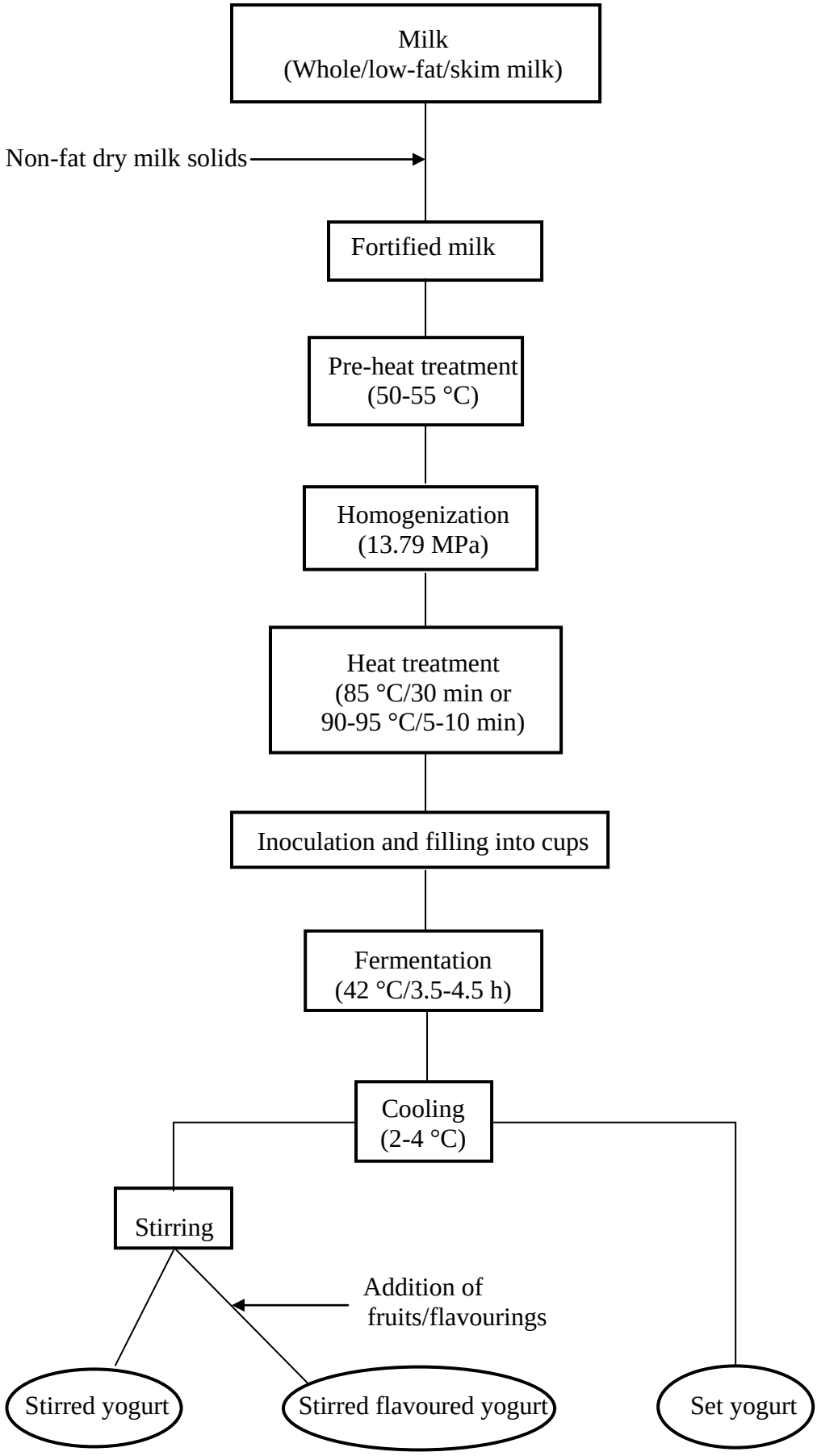

Figure 2.1 Major steps in yogurt making.

The proteolytic activity of LAB could also exert an effect on the formation and stability of the milk protein gels (Laws and Marshall, 2001), but it is not clear if proteinase activity of LAB actually affects the structure of fermented milk. Enzymatic hydrolysis of casein caused by proteases from psychrotrophic bacteria, or by native milk plasmin, is known to give yogurts with different firmness, viscosity and degree of syneresis (Gassem and Frank, 1991). Shihata and Shah (2002) reported that addition of proteolytic strains of *L. delbrueckii* ssp. *bulgaricus* to commercial ABT (*L. acidophilus*, *Bifidobacterium* and *S. thermophilus*) cultures improved the fermentation times and viability of probiotics in the yogurt and also improved the firmness of the yogurts.

In recent times, the fat content of dairy products is being reduced to lower the daily energy intake and thereby improve the energy balance of the consumers. Since milk fat has an important role in the texture, flavour and colour development of dairy products, the removal or reduction of fat can cause some defects in such products, including yogurt, such as lack of flavour and mouth-feel, weak body and poor texture. Regardless of the widespread success of low-fat and no-fat yogurts, quality problems such as weak body and poor texture still exist because of the lower total solids content of these yogurts. It is more challenging to control the texture of low-fat and no-fat yogurts because the presence of homogenized fat which contributes to the structure of yogurt is either absent or in very low concentrations in these products.

Table 2.6 Benefits of fortification and heat treatment of yogurt milk base.

Step	Additive	Observed effects	Reference
Increasing total solids	Skim milk powder / skimmed milk concentrate / Butter milk powder	Better growth of culture	Ozer and Robinson (1999); Mahdian and Tehrani (2007)
		Higher water holding capacity; Increased firmness; Increased viscosity	Harwalkar and Kalab (1986); Hess et al. (1997)
		Reduced syneresis	Mahdian and Tehrani (2007)
Addition of proteins	Whey protein hydrolysate	Improved growth of probiotics	McComas and Gilliland (2003)
	Whey protein concentrate	Improved water holding capacity	Guinee et al. (1995); Remeuf et al. (2003)
		Increased firmness	Cheng et al. (2000); Dave and Shah (1998b)
		Increased viscosity	Guzmán-González et al. (2000); Remeuf et al. (2003)
	Sodium caseinate	Increased firmness	Tamime et al. (1984)
		Increased complex viscosity	Lankes et al. (1998)
		Decreased fermentation time	Damin et al. (2009)
		Increased yield stress	
	Whey protein powder	Increased viscosity	Guzmán-González et al. (2000)
		Reduced syneresis	Guzmán-González et al. (2000)
		Improved shelf life	González-Martinéz et al. (2002)
	Whey protein isolate	Increased viscosity; Reduced syneresis	Mistry and Hassan (1992); Isleten and Karagul-Yuceer (2006)
High heat treatment	-	Increased gel firmness	Lucey et al. (1998b); Vasbinder et al. (2004)
		Reduced syneresis	Lucey et al. (1998b)
		Better rate of gelation	Xu et al. (2008)

2.3.6 Texture and rheological properties of yogurt

2.3.6.1 Rheological properties of food

Rheological properties of foods, such as fermented dairy products, are important in the design of flow processes, quality control, storage and processing and in predicting the texture of foods (Shaker et al., 2000). According to the International Standards Organization, texture of food products represents all the rheological and structural attributes perceptible by means of mechanical, tactile, and, when appropriate, visual and auditory receptors. Hence, texture is related to sensory perception of food product. However, rheology and structure of a product evaluated by instrumental methods also give relevant information on its textural properties, even though sensory and instrumental data are not always easily correlated (Sodini et al., 2004).

Rheology is the study of the flow and deformation of matter. In food research, the term is often used inter-changeably with texture, which refers to the flow, deformation, and disintegration of a sample under force. Strictly speaking, texture relates to solid foods, and viscosity, the tendency to resist flow, relates to fluid foods. However, food can exhibit both solid and liquid characteristics, and rheology can identify the properties of such food (Tunick, 2000). 'In a dynamic rheological experiment, popularly known as small amplitude oscillatory tests, a sinusoidal oscillating stress or strain with a frequency ω is applied to the material and the phase difference between the oscillating stress and strain as well as the amplitude ratio is measured' (Rao, 2003). As a result of the applied strain, two stress components are generated in the viscoelastic material, an elastic component that is in line with the strain and the viscous component that is out-of-phase (90°). For deformation within the linear viscoelastic range, the generated stress can be measured in terms of an elastic or storage modulus G´ and a viscous or loss modulus G´´. The storage modulus expresses the magnitude of the energy that is stored in the material or recoverable per cycle of deformation. The loss modulus is a measure of the energy which is lost as viscous dissipation per cycle of deformation (Rao, 2003).

2.3.6.2 Texture and rheological measurements in yogurt

Texture is one of the most essential components of yogurt quality and hence, gelation is a critical step in yogurt manufacture. The important physical attributes that contribute to the overall sensory perception and functionality of yogurt include overall visual appearance, microstructure and rheological properties of the gels.

In terms of rheology, ‘yogurts are non-Newtonian highly structured materials that behave as very weak gels’(Harte et al., 2007). All undisturbed yogurts behave as viscoelastic materials. The structure of yogurt is the result of disulfide bonding between κ-casein and denatured whey proteins and the aggregation of caseins as the pH drops to the isoelectric point of the caseins (~ pH 4.6) during fermentation that forms a continuous colloidal network. This structure is extremely fragile and can easily be disrupted if the gel matrix is subjected to mechanical disturbance (Harte et al., 2007).

The non-Newtonian effects exhibited by yogurt include shear thinning, yield stress, viscoelasticity, and time-dependency. An important characteristic of the product is the profile of the value of viscosity obtained for different shear rates. The viscosity of fermented milk products is often observed to decrease with increasing shear rates. This means they are shear-thinning. In other words, the more vigorous the agitation conditions, the more fluid the fermented product. This is meaningful for the mouth-feel of a texturized product, since shear rates applied during chewing and swallowing are about 30-50 s^{-1} (Duboc and Mollet, 2001).

The rheological characterization of yogurt gels requires at least two kinds of measurements to define viscoelasticity and flow properties. Rheometers working in dynamic mode are used to calculate storage and loss modulus that describe the elastic and viscous properties of the gelling system, respectively (Sodini et al., 2004). The dynamic response of a viscoelastic fluid to an oscillatory stimulation results from two contributions: the storage modulus G´ and the loss modulus G´´, corresponding to elasticity and viscosity, respectively. Additionally, complex viscosity and the ratio between loss and storage modulus are also used to describe the rheological properties of yogurt gels. Another important rheological property is thixotropy, ‘the ability to recover the original structure after cessation of a shearing action’ (Duboc and Mollet, 2001). The firmness of the gel can be measured by the force required to push a probe into yogurt at a fixed depth of penetration. Yogurt gels also exhibit a ‘yield value’ which is ‘the point at which the applied stress exceeds a certain value and the fluid deforms/flows’ (Laws and Marshall, 2001).

2.3.6.3 The problem of whey separation

Whey or serum separation, which is also called wheying off, ‘is the appearance of whey on the surface of a gel’ (Lucey, 2002), and is a common defect during gelation and subsequent storage of fermented products such as yogurt. ‘Spontaneous syneresis is the contraction of a gel without the application of any external forces (e.g. centrifugation)’ (Lucey, 2002). Casein gels are dynamic by nature. Excessive rearrangements of particles making up the gel network before and during gelation have been considered to be responsible for whey separation. Hence, whey separation is related to the instability of the gel network that has a strong tendency to undergo further rearrangement of network structure resulting in loss of the ability of the gel to entrap all the serum phase. Conditions under which considerable whey separation could occur include high incubation temperature (45 °C), excessive pre-heat treatment (> 80 °C for 30 min), disturbances while the gel is still weak, low acid production (pH 4.9 instead of 4.6) and low total solids content (Lucey, 2002; Xu et al., 2008).

Although heat treatment increases the rigidity of yogurt gels (which is an important textural attribute), it is not very effective in preventing the wheying-off that occurs in milk incubated at very high temperatures (Lucey et al., 1998b). In acid gels made from heated milk, there is an increase in loss tangent during gelation even at much higher frequencies and a reduction in fracture strain both of which could assist in rearrangements and whey separation (Lucey, 2001). However, Al-Kadamany et al. (2003) reported that samples of labneh stored at 5 °C syneresed to a lower degree, and displayed marked fluctuations in the degree of syneresis, as compared to those stored at 15 and 25 °C. Thus, the susceptibility of yogurt gels to whey separation varies markedly and is poorly understood. Some rheological parameters of the gel network that may possibly indicate rearrangements that could lead to instability of acid milk gels are given in Table 2.7.

Table 2.7 Rheological parameters indicating rearrangements of interactions within acid milk gels.

Parameter	Definition	Indicators of rearrangement
Elastic or storage modulus (G´)	Energy stored per deformation cycle during an oscillatory test; related to the stiffness of the network	Low G´values for the final gel indicate that strength and number of bonds in the network may be low enough to be easily broken by the stresses in the network caused by ongoing fusion of particles and/or strands
Loss tangent	Ratio of storage to loss moduli (tan δ = G´/G´´), indicates the viscoelastic character of the material (e.g. more solid-like or liquid-like)	Higher values favour relaxation of bonds
Fracture stress	The value of the shear stress at which the gel network starts to breakdown	Determines the susceptibility of the strands to breakage; a low value of indicates a weak or soft gel
Fracture strain	The value of the strain at which point the network starts to breakdown	Determines the susceptibility of the strands to breakage; low values indicate a brittle or short texture

(Source: Lucey, 2001)

For syneresis to occur, a combination of several of these conditions must be met, e.g., a low value for the storage modulus and a low fracture stress.

2.4 Role of Exopolysaccharides (EPS) in Texture of Yogurt

The most important textural characteristics of yogurt are firmness and the ability to retain water. These properties are related with the gel structure and can be influenced by the type of culture (Ruas-Madiedo et al. 2002). There is a high consumer demand for smooth and creamy yogurt products, which is typically met by increasing the content of fat, sugars, proteins or stabilizers (e.g. pectin, starch, alginate or gelatine). The amounts of these ingredients required to achieve the total solids content similar to full-fat yogurt can lead to a powdery taste, excessive acid development from lactose fermentation, excessive firmness, higher whey expulsion, and grainy texture (Mistry and Hassan, 1992; Guzmán-González et al., 2000). The polysaccharides produced by LAB, considered as ‘food-grade’ additives, provide a viable alternative for producing a creamy low-fat yogurt when considering consumer demand for products with low fat or sugar content and low level of additives, as

well as cost factors (Jolly et al., 2002). Yogurt manufacture remains the most important commercial application of EPS in dairy foods.

2.4.1 EPS produced by lactic acid bacteria

Lactic acid bacteria are able to produce several types of polysaccharides, classified according to their location relative to the cell. Those that are excreted outside the cell wall are called exocellular polysaccharides (EPS) while those that form an adherent cohesive layer are called capsular polysaccharides. The EPS can be either loosely attached or be completely excreted into the environment as slime. Apart from the physiological and ecological functions outlined in Table 2.8, EPS from LAB have technological significance in the production of several fermented dairy products (Ruas-Madiedo et al., 2002).

Table 2.8 Ecological and physiological functions of EPS produced by LAB.

	Function	Reference
Ecological function	Protection against desiccation, phagocytosis and predation by protozoa, phage attack, antibiotics or toxic compounds, osmotic stress, metal ions, lysozymes	DeVuyst and Degeest (1999); Looijesteijn et al. (2001)
	Cell recognition, adhesion to surfaces, formation of biofilms (facilitates colonization in various ecosystems)	DeVuyst and Deegest (1999)
Physiological functions	Prebiotics	Hugenholtz and Smid (2002)
	Antitumor	Kitazawa et al. (1998)
	Antiulcer and immunomodulating	Hosono et al. (1997); Chabot et al. (2001)
	Cholesterol lowering	Nakajima et al. (1992)
	Enhanced colonization of probiotics	German et al. (1999)

The amount of EPS produced in milk varies with the strain and species of the cultures used, and also depends on the EPS isolation method employed. Growth conditions (pH, temperature and incubation time) and medium composition (carbon, nitrogen sources

and other nutrients) can affect the yield and the sugar composition of EPS produced. The quantity of EPS produced by lactic cultures ranges from 50 to 350 mg/L for *S. thermophilus*, from 60 to 150 mg/L for *L. delbrueckii* ssp. *bulgaricus*, from 25-600 mg/L for *L. lactis* ssp. *cremoris* and from 50 to 60 mg/L for *L. casei* (Cerning, 1995). The concentration of EPS in milk cultures has been reported to go up to 3000 mg/L for *S. thermophilus*, to 2100 mg/L for *L. delbrueckii* ssp. *bulgaricus*, to 490 mg/L for *L. casei*, and to 600 mg/L for *L. lactis* ssp. *cremoris* by modifying medium composition and growth conditions (Duboc and Mollet, 2001). Zisu and Shah (2003) have suggested the use of non-EPS producing adjunct *S. thermophilus* along with EPS-producing *S. thermophilus* as a means of increasing the production of EPS. Mozzi et al. (2006) evaluated the EPS production in milk by 31 LAB strains from different species and observed that thermophilic strains produced more EPS than mesophilic ones, but EPS yields were generally low. Ropiness or capsular polysaccharide formation was strain dependent. Recently, Law et al. (2009) have reported that during the exponential growth phase of *L. acidophilus* 5e2 the increase in molecular mass of EPS secreted closely followed the increase in yield of EPS. They found that the increase in yield during the growth, exponential and stationary phases was accounted for by an increase in chain length of the EPS secreted.

Exopolysaccharides from LAB are tasteless but their presence increases the time the milk product spends in the mouth, and hence imparts an enhanced perception of taste (Duboc and Mollet, 2001). Thus, incorporation of isolated EPS or EPS producing cultures can provide viscosity, stability, and water-binding functions (De Vuyst and Degeest, 1999) that may contribute positively to the mouth-feel, texture and taste perception of yogurt (Duboc and Mollet, 2001).

2.4.2 Contribution of EPS to texture related properties and structure of yogurt

Bacterial EPS influence the rheology and texture of fermented products at extremely low concentrations and favourably compares with other thickeners. Due to the complex physico-chemical process involved in texture generation, the mere ropiness trait of a culture strain does not guarantee an optimal, smooth and creamy quality of the yogurt (Duboc and Mollet, 2001). Use of EPS-producing cultures can improve textural properties in low-fat yogurts and can be used instead of using additives such as fat replacers (Guzel-Seydim et al., 2005). The use of ropy starters containing EPS-forming *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* is a common practice in the production of yogurt to improve texture, avoid syneresis and increase the viscosity of yogurt. Most of the research regarding the use EPS-

producing cultures has studied their influence on the firmness, whey separation, microstructure and rheological properties of yogurt as described below.

2.4.2.1 Firmness and gel structure

Hess et al. (1997) concluded that the EPS produced, or the interaction of EPS with the bacterial cells, milk proteins, or both is different from that of commercial stabilizers. In general, they observed that yogurts made using EPS-producing strains of *L. delbrueckii* ssp. *bulgaricus* required less force to penetrate the gel, had lower power law index and exhibited less shear thinning, all of which indicated that a weaker coagulum was formed as compared to yogurts made with strains not producing EPS. However, they suggested that separation of EPS from the bacterial cells may result in the formation of new sticky ends on the EPS strands where aggregation could occur, counteracting the effects of structural degradation.

The microstructure of yogurt consists of a matrix of aggregated casein particles that changes according to the type of organism used. Duboc and Mollet (2001) reported that fat globules were embedded in the yogurt matrix and that the cavities of the gels were filled with serum and bacterial cells. An envelope of EPS was observed surrounding the bacterial starter strains, by which ropy cells attach to the protein matrix via a web of filaments. Further, the attachment of cells to the protein matrix was more pronounced in set-type yogurts than in stirred products. Differences were observed in the microstructure of yogurt products manufactured with ropy (i.e. with EPS-producing) and non-ropy strains where the protein gels obtained with the ropy strains showed a homogenous structure with randomly distributed small cavities whereas those obtained with non-ropy strains showed larger cavities filled with bacteria and serum.

2.4.2.2 Viscoelastic properties and gel structure

In general, firmness and cohesiveness of the gel structure decreased when ropy bacteria were used, the decrease being more with the presence of increased amounts of EPS. Rawson and Marshall (1997) found that ropy starter bacteria increased yogurt viscosity, but firmness or elasticity was not always increased. The same study also found that yogurt made with a nonropy *S. thermophilus* and a ropy *L. delbrueckii* ssp. *bulgaricus* recovered its viscosity more rapidly after destructive testing than yogurt made from two ropy cultures. The authors suggested that this effect was due to EPS interference with protein bonding, which led to formation of larger voids within the protein matrix. Studies of yogurt microstructure (Hassan et al., 1995) showed void spaces around EPS-producing bacteria in confocal

scanning laser micrographies that can affect the integrity of the protein matrix. Using cryo-scanning electron microscopy, Hassan et al. (2003a) observed a compact well-defined protein network in milk fermented with EPS non-producing culture and an open structure in yogurt made with EPS-producing culture. Exopolysaccharides partially or completely filled pores within the structure. Milk fermented with moderately ropy strain of *S. thermophilus* CHCC3534 contained larger pores than that made with the highly ropy strain of *L. lactis* ssp. *cremoris* JFR1. In both cases EPS appeared as large clusters segregated from the protein network. Higher magnification of the structures revealed that the EPS produced by *S. thermophilus* formed a well-defined porous network, while that produced by *L. lactis* ssp. *cremoris* had a more dense entangled appearance and contained randomly distributed relatively thicker filaments.

The polysaccharides of different LAB greatly vary in their molar mass, monosaccharide composition, and linkage type; charge, spatial arrangements, rigidity, and ability to interact with proteins. Hence, no clear correlation between observed EPS concentrations and apparent viscosities of the product could be established (Jolly et al., 2002). van Marle and Zoon (1995) also could not find a simple correlation between ropiness and the amount of EPS produced from cultures where the EPS produced were similar, consisting of the same sugar residues but in different ratios. This finding was supported by Sebastiani and Zelger (1998) for strains of *L. delbrueckii* ssp. *bulgaricus, S. thermophilus* and *S. filant.* Bouzar et al. (1997) looked at different mixed strain starter cultures for their ability to influence texture of yogurt and found that consistency (viscosity and smoothness) of the milks could be improved if ropy strains were used. Kalab et al. (1983), Tamime et al. (1984) and Schellhaass and Morris (1985) suggest that EPS filaments attach mucoid bacteria to the protein matrix, thus causing more viscous-like behaviour. However, Ruas-Madiedo et al. (2005) reported that the yield of EPS and the timing of EPS production by strains of *L. lactis* ssp. *cremoris*, during milk gel formation, were the most important factors that influenced the structure of the milk gels and the viscosity of the stirred product. The proteolytic activity of the strains did not seem to play any significant role.

Based on permeability measurements and confocal scanning laser micrographs, it is suggested that the spatial structure of the protein network is not the only factor that influences the apparent viscosity of the stirred yogurt. Marshall and Rawson (1999) found that combining a non-ropy strain of *S. thermophilus* with a ropy strain of *L. delbrueckii* ssp. *bulgaricus* had a greater effect on viscosity of stirred yogurt than combining two ropy strains. It is supposed that differences in the ability of various strains to enhance viscosity are

due to differences in the intrinsic viscosity of the EPS. To obtain a high viscosity, the molar mass should be high and the chain should be relatively stiff (Ruas-Madiedo et al., 2002). Folkenberg et al. (2006a) found that a strong protein network, not too dense and with medium size pores containing EPS were associated with microstructural characteristics resulting in sensory characteristics such as good mouth thickness, creaminess and viscosity. Furthermore they observed that the presence of capsular EPS was as beneficial as EPS present in pores or in association with protein. They also observed that charged EPS interact with the protein network while those lacking charge were found in the pores of the network.

2.4.2.3 Rheological properties and gel structure

The effect of EPS on the rheological properties and the microstructure of yogurt can, to a large extent, be explained by its incompatibility with the protein aggregates in the product. This incompatibility probably affects the aggregation prior to gelation, as well as the rearrangement of the protein aggregates after the gel point, resulting in a network with a microstructure composed of rather thick, aggregated protein strands interspaced with pores containing EPS in the unstirred product. The increased consistency index and deviation from Newtonian flow of stirred yogurt may also be influenced by the effects of incompatibility on structure but are probably affected directly by the rheological properties of the EPS as well. Further, Ruas-Madiedo and Zoon (2003) postulated that the time at which EPS is produced during gel formation could influence the flow properties of the serum and thereby the permeability of the gels. Hassan et al. (2003b) observed that yogurt made with EPS-producing strain of *S. thermophilus* exhibited increased consistency coefficients, but lower flow behaviour index, yield stress, viscoelastic moduli and phase angle values than did yogurt made with the culture unable to produce EPS. The EPS were found in pores in the gel network, separate from the aggregated protein which is due to the incompatibility of the EPS with the protein aggregates in the milk.

Folkenberg et al. (2006b) observed that a high degree of hysteresis in EPS containing yogurts indicates that these products have a lower ability to regain structure after shear induced structure breakdown. According to their hypothesis, in the rheometer, as well as in the mouth during eating, the structure is partly destroyed (viscosity decreases with increasing shear rate). Thus, during the shear treatment EPS can be expected to be redistributed in large clusters into the interstitial continuous phase surrounding the acid casein gel particles resulting from fracture of the original gel. Due to the incompatibility between casein and the EPS, the interstitial phase would hinder reassociation of curd fragments. In EPS-free

yogurts, however, this reassociation can be expected to occur faster and to resemble the original structure much more. A higher level of hysteresis is therefore expected in the EPS containing yogurts.

2.4.3 EPS and syneresis of yogurt gels

Exopolysaccharides have also been shown to act as a stabilizer that retains water and hence decreases susceptibility of yogurt to syneresis. La Torre et al. (2003) found that the type of starter culture used affected the rate of initial syneresis in set-type probiotic yogurt. Yogurts made with ropy cultures exhibited highest water holding capacity which decreased susceptibility to syneresis (Hassan et al., 1996b; Ruas-Madiedo et al., 2002). Wacher-Rodarte et al. (1993) observed that when EPS producing strains were used, the increase in water holding capacity of yogurt due to total solids enrichment was not significant. Amatayakul et al. (2006a) reported that the physical characteristics of set yogurt can be improved by varying casein-whey protein ratio and by the use of EPS-producing starter cultures. A decrease in the ratio of casein-whey protein and the use of EPS-producing starter cultures reduced the level of syneresis and firmness. Guzel-Seydim et al. (2005) also observed that whey separation in yogurt decreased more with ropy cultures when fermented at 35 °C than with non-ropy cultures at the same incubation temperature. Purwandari et al. (2007) observed that yogurt made only with EPS producing strain of *S. thermophilus* resulted in reduced syneresis while Aryana et al. (2007) observed lower syneresis and higher survival of *L. acidophilus* in probiotic yogurt than the corresponding control during 21 d storage at 4 °C.

The shear-induced microstructure in yogurt made with EPS-producing culture was shown to consist of compartmentalized protein aggregates between channels containing EPS, hindering syneresis as well as the buildup of structure after stirring and also caused the yogurt to have lower moduli (G´ and G´´) and yield stress (Hassan et al., 2003b). However, the beneficial effects of increased EPS were not always apparent as the EPS from *S. thermophilus* was reported to result in a greater susceptibility to syneresis (Laws and Marshall, 2001).

2.5 Fat Replacers in Low-fat Foods

Fats in foods have three basic physiological functions as:

a. a source of essential fatty acids;
b. carriers for fat-soluble vitamins and
c. an important source of energy.

Functionally, fat affects the physico-chemical properties of the product and is therefore involved in the behaviour of the food product during processing, post-processing characteristics and storage stability. It also determines the sensory characteristics of food products, mainly appearance, texture, flavour and mouth-feel. However, changing lifestyles of mankind over the years have substantially decreased the requirement for energy from food resulting in an increase in the demand for low- or no-fat foods. Considering the various roles of fat in foods, it becomes important that the low-fat variants of the food match the quality of the full-fat counterparts which will thus depend on the replacing fat with an alternative ingredient. Many ingredients have been developed for the specific purpose of fat replacement in foods.

2.5.1 Terminology and classification of fat replacers

The terms used to describe various ingredients which can replace fat in foods are:

a. Fat replacer: This term is used to describe any ingredient used to replace fat.
b. Fat substitute: A synthetic compound designed to replace fat on a weight-by-weight basis, usually having a similar chemical structure to fat but resistant to hydrolysis by digestive enzymes. They have similar physicochemical properties as fat and contribute lower calories on a per gram basis.
c. Fat mimetic: This term refers to a fat replacer that requires high water content to achieve its functionality. They have distinctly different chemical structures from fat. They are usually carbohydrate or protein based. They have diverse functional properties that mimic some of the characteristic physicochemical attributes and desirable eating qualities of fat: viscosity, mouthfeel and appearance. They are the most widely used ingredients for producing emulsion-based reduced-fat product.
d. Low-calorie fat: A synthetic triglyceride combining unconventional fatty acids to the glycerol backbone which results in reduced caloric value

e. Fat extender: This is a fat replacement system containing a proportion of standard fats or oils combined with other ingredients (Singer, 1996; Johnson, 2000).

Based on chemical composition, functionality of ingredients and the combination thereof, fat replacers may be classified as follows:

a. Starch-derived
b. Fibre-based
c. Protein-based
d. Gums, gels and thickeners
e. Emulsifiers
f. Bulking agents
g. Low-calorie fats
h. Fat extenders
i. Synthetic fat substitutes
j. Combination systems.

This review focuses on protein- and fibre-based fat replacers.

2.5.1.1 Protein-based fat replacers

Two decades ago protein-based fat replacers were introduced as useful ingredients in low-fat foods (www.fda.gov). They include microparticulated protein (MPP) derived from milk, egg, whey or vegetable proteins such as soy. The most popular form of MPP starts with whey protein concentrate (WPC), using either 35 or 50% WPC. The use of MPP helps retain the traditional sensory qualities of foods while substantially reducing their fat content (Singer, 1996). Additionally, MPP is reported to be stable towards pH (pH 3 to 7) and heat (10 to 95 °C).

Whey protein concentrates, also considered as fat mimetics, have found extensive use in reduced-fat foods, either alone or in combination with other mimetics. Typically WPC34 and WPC80 (whey products containing 34% and 80% protein, respectively) are used as fat mimetics. Their major functions are gelling, water binding, emulsification, viscosification and adhesion, which can be selectively enhanced by modifying processing conditions during the manufacture of WPCs. They retain their functionality in low pH environments and provide benefits such as cost reduction, improved texture, mouthfeel and superior nutritional profile (Johnson, 2000).

Some of the protein-based fat replacers available in the market that are suggested for use, particularly in dairy products, are given in Table 2.9.

Table 2.9 Some commercially available protein-based fat replacers for use in dairy products.

Trade/ Common name	Chemical name/Composition	Developer/Manufacturer/ Supplier	Applications
Dairy-Lo, Dairy light	Whey protein concentrate – partially denatured	Ault Foods Ltd., Canada	Frozen desserts, ice cream, yogurt, cheese, spreads, sour cream
Lactomil	Partially denatured milk protein	MILEI, Germany	Dairy based products: butter, frozen desserts, cream spreads, sour cream, ice cream
Lita	Microparticulated zein protein	Opta Food Ingredients Inc., MA, USA	Frozen desserts, milk, yogurt, cream/cottage cheese, dips, chocolate and confectionery
Nutrilac YO	Whey protein concentrates obtained through extrusion	Denmark Proteins A/S, Denmark, Royal Proteins Inc., IL, USA	Yogurt and cultured products
Simplesse 100, D-100, D-500	Whey protein concentrate – microparticulated protein	Nutrasweet Company, IL, USA	Frozen desserts, dairy products, baked goods, ice cream
Trailblazer range	Microfragmented protein formed through protein/ polysaccharide interaction	Kraft General Foods, IL, USA	Frozen desserts, Spreads, cheese products, cultured dairy products, baked goods
Versagel	Whey protein gel	Food Science Australia	Reduced fat bakery and dairy products

(Source: Jones, 1996b)

2.5.1.2 Fibre-based fat replacers

A number of fat replacers based on fibre from a number of different sources such as oats, sugar beet, including inulin, have been launched in the market. The increasing recognition for the role of dietary fibre in disease prevention, particularly in relation to colonic cancer and heart disease has endowed certain physiological benefits to these ingredients (Jones, 1996a). Some of the fibre-based fat replacers available in the market that are suggested for use, particularly in dairy products, are given in the Table 2.10.

Table 2.10 Some commercially available fibre-based fat replacers for use in dairy products.

Trade/Common name	Chemical name/Composition	Developer/Manufacturer/Supplier	Applications
Avicel	Microcrystalline cellulose, alginate salts	FMC Corporation, PA, USA	Bakery products, sauces, salad dressings, dips, spreads, dairy products, ice cream and frozen desserts, meat products
Better Basics Advanced Oat Fibre	Oat fibre	Williamson Fobre Products, Ireland	Processed meats, ice cream, batter coated products and deep fried food, chocolate, mayonnaise, spreads, frozen yogurt, Danish pastry
Fibercel	Beta-glucan fibre derived from yeast	Alpha-beta Technology, MA, USA	Ice cream, frozen yogurt, cheese products, processed meats, puddings, mayonnaise, salad dressings, frostings and icings, fillings, beverages soups, canned foods, breads
Fibruline	Inulin	Cosucra SA, Momalle, Belgium	Ice cream, cheese spreads, chocolate dressings, meat products
Justfiber	Cellulose/cottonseed fibre	Ingredients Groups, IL, USA	Dairy products, salad dressings, bakery products, beverages
Raftiline (now known as Beneo)	Inulin obtained from chicory root	Orafti, Belgium	Bakery products and ingredients, dairy products, desserts, spreads, ice cream, yogurt, soft cheese

(Source: Jones, 1996b)

Of these, inulin has found extensive use due to their health-promoting and technological properties.

2.5.1.2.1 Inulin

The industrial extraction of inulin-type fructans is commonly done from plants that belong to the *Compositae* family, i.e., chicory. The degree of polymerization (DP) of chicory inulin varies from 2 to ~ 60 units. High molecular weight inulin, also known as high performance inulin (inulin HP), is prepared by applying physical separation techniques to eliminate all oligomers with DP < 10. Inulin HP has an average DP of 25 and a molecular distribution range from 11 to 60 (Roberfroid, 2007).

Inulin is used either as supplements to foods or as macronutrient substitutes in foods. As supplements to foods, it is added mainly for its nutritional properties. Such additions are usually in the range of 3-6g per portion, not exceeding 10g. As macronutrient substitutes, it is used mainly as a fat replacer. Typically 1 g of fat is replaced by 0.25g of inulin which will lead to inulin concentrations of ~ 6g per portion. Since inulin has been a natural component of many foods consumed safely by humans over millennia, it is therefore generally recognized as safe (Coussement, 1999).

Inulin-type fructans are classified as functional food ingredients. They are believed to target gastrointestinal functions and also, most likely via their effects on the gut and the gut microflora, systemic functions that are known to be related to health and well-being (Roberfroid, 2007). The potential nutritional and health benefits of this ingredient are summarized in Table 2.11.

Inulin has a neutral taste, is colourless and has minimal influence on the organoleptic characteristics of a product (Niness, 1999). Solubility of inulin increases significantly with temperature, reaching 34% (w/v) at 90 °C (Kim et al., 2001). The functionality of inulin depends on its effect on water solutions at various solid levels. At lower concentrations it can be used as a rheology modifier since it causes significant increase in viscosity while at a concentration of 40-45% an inulin gel or crème is formed which is firm but with a fatty creamy feel. In this form inulin is stable in acidic conditions and at high temperatures (Murphy, 2001).

Table 2.11 Nutritional effects and potential health benefits of inulin-type fructans.

Enhanced colonic functions	Composition and activities of the gut microflora
	Stool production
	Absorption of minerals
	Production of gastrointestinal endocrine peptides
	Immunity and resistance to infections
	Digestion of high protein diets
Enhanced systemic functions	Lipid homeostasis
Reduction of disease risks	Intestinal infections
	Irritable bowel disease
	Colon cancer
	Osteoporosis
	Obesity
	Diabetes

(Source: Ninesse, 1999; Roberfroid, 2007)

Long-chain inulin is stable at low pH and heat, less soluble and more viscous than the native product and can be used as a fat substitute, with an efficiency that is practically double than that of native inulin. It also does not show any reducing capacity thus indicating that it does not participate in Maillard reaction. However, heating at low pH reduces its prebiotic activity (Villegas and Costell, 2007; Huebner et al., 2008). Inulin HP provides almost twice the fat mimetic characteristics of standard inulin without contributing to sweetness. It is less soluble because of its long chain length, and has the ability to form inulin microcrystals when sheared in water or milk. These crystals are not discretely perceptible in the mouth, but they interact to form a smooth creamy texture and provide a fat-like mouthfeel (Ninesse, 1999). Thus, it can be used in fat-free products and can replace

fat having an energy value of 37.6 kJ/g with an inulin/water combination which has an energy value of 2.09 kJ/g or less, resulting in significant calorie reduction (Murphy, 2001).

2.5.2 Role of fat replacers in low-fat yogurt

Fat solids reduction in yogurt has been associated with poor texture, flavour and mouth feel. One approach to solve this problem is by replacing partially the fat content of the milk base with fat replacers.

2.5.2.1 Protein-based fat replacers

Very little work has been carried out to study the influence of commercially available protein-based fat replacers on textural properties of low- or no-fat yogurt, as summarized below.

Tamime et al. (1995) studied the effect of the fat replacer Simplesse® 100 (MWP) and found that it resulted in higher serum separation and lower firmness of set-style yogurts containing MWP than those containing anhydrous milk fat. They observed that MWP and anhydrous milk fat became an integral part of yogurt microstructure, but the fat replacer particles appeared to be larger than the milk fat globules which explained the textural defects observed in yogurt containing MWP.

Sandoval-Castilla et al. (2004) showed that the protein matrix of reduced-fat yogurts made with and without fat replacers (DairyLo®; Simplesse 100 and their combination), showed differing structures, which in general terms were more open and less dense than that of the full-fat yogurt. Casein micelles were predominantly linked by particle-to-particle attachment in long chains in yogurts containing DairyLo, whereas yogurt containing Simplesse 100 (MWP) showed a spatial distribution of casein micelles similar to that of reduced-fat yogurt, with the ingredient forming part of the protein matrix. Yogurts with blends of DairyLo and Simplesse showed textural characteristics resembling those of full-fat yogurt, whereas yogurt with Simplesse showed lower tension and firmness but higher cohesiveness.

Akalin et al. (2007) used WPC (1.5%, w/v) in reduced-fat probiotic yogurt. They observed that addition of WPC to yogurt increased the buffering capacity around pH 4 which controlled the progress of acidification during storage. They reported that this effect of WPC on slow acidification in probiotic yogurt contributed to the enhanced shelf life of the product. Whey protein concentrate supplemented yogurt exhibited improved viability and

survival rate of *S. thermophilus* and *B. animalis* until the 21st day of storage. They attributed the growth promoting activity obtained by WPC to its whey protein content.

2.5.2.2 Fibre-based fat replacers

The limited work carried out over the past decade that studied the influence of inulin on the physico-chemical and textural properties of yogurt has been outlined below.

Guven et al. (2005) reported that the use of inulin (Raftiline HP) as a fat replacer did not affect the pH values but negatively influenced some physical properties such as whey separation, consistency and organoleptic properties of fat-free yogurt. They found that the consistency was highest in yogurt containing 1% inulin.

Özer et al. (2005) observed that the addition of inulin (0.5%) reduced proteolysis development in yogurt samples. It also caused a substantial increase in the cell counts of *B. bifidum* BB-02, being about 4.6 and 7.5 fold for the inulin added samples at levels of 0.5% and 1.0%, respectively. They concluded that supplementation of milk with inulin as growth promoters for *B. bifidum* BB-02 and *L. acidophilus* LA-5 could be a satisfactory way of keeping the number of viable probiotic cells in AB yogurt above the suggested therapeutic minimum (~10^7 CFU/g) during cold storage.

Kip et al. (2006) found that inulin (DP 23, 3%) contributed to an improved creamy mouthfeel by enhancing the attribute airy and having a positive effect on thickness and stickiness.

Aryana et al. (2007) demonstrated that the chain length of inulin (small, medium or long) affected the pH and syneresis in yogurt and that inulin helped the survival of *L. acidophilus* in the product.

2.6 Health Benefits of Yogurt

2.6.1 Nutritional and health benefits of yogurt

In terms of its nutritional profile, yogurt has a similar composition to the milk from which it is made but will vary somewhat if fruit, cereal or other components are added. The nutritional similarity of yogurt with milk implies that it is an excellent source of protein, calcium, phosphorus, riboflavin, thiamine and vitamin B_{12}, and a valuable source of folate, niacin, magnesium and zinc. The protein it provides is of high biological value and the vitamins and minerals are bioavailable. Moreover, the low-fat varieties of yogurt provide an

array of important nutrients in significant amounts in relation to their energy and fat content, therefore making them a nutrient-dense food (McKinley, 2005).

Metchnikoff suggested that the LAB in yogurt were responsible for longevity of its consumers (Shah, 2007). Thus, cultured milk products such as yogurt have been used for a very long time and for many reasons, one of which was for its perceived health benefits mainly associated to the presence of LAB that could retard the growth of other harmful bacteria. In the last few decades a major development in the functional food sector has emerged from the use of probiotic bacteria and prebiotic carbohydrates that enhance health-promoting microorganisms in the intestine. To realize the health benefits, probiotic bacteria must not only be viable and available in a high concentration, typically 10^6 CFU/g of a product, but also be able to survive passage through the harsh conditions of the GIT so that they reach their target site in live form (McKinley, 2005; Shah, 2007).

The main health benefits of regular consumption of probiotic containing products include the improvement of the intestinal microbial balance; alleviating the symptoms of lactose intolerance through the production of lactase; strengthening the immune system; reducing the risk of colon cancer in human studies and protection against breast cancer; reducing some forms of food allergies; lowering the blood cholesterol levels; suppression of blood pressure of hypertensive individuals; playing a key role in the prevention of diarrhoea; and inhibiting the growth of some pathogenic bacteria (Alhaj et al., 2007). Health benefits imparted by probiotic bacteria are strain specific, not species- or genus-specific. No strain can provide all proposed benefits, and not all strains of the same species can be effective against defined health conditions. The strains of *L. rhamnosus* GG, *S. cerevisiae*, *L. casei* Shirota, and *B. animalis* have the strongest human health efficacy data with respect to management of lactose malabsorption, rotaviral diarrhoea and antibiotic-associated diarrhoea (Shah, 2007).

2.6.2 Release of bioactive peptides as a consequence of proteolysis

2.6.2.1 Proteolytic activity of LAB

Energy and nitrogen are required by the yogurt starters to maintain their life cycle. Lactose in milk is the only available carbohydrate for providing energy to LAB that possesses the enzyme lactate dehydrogenase for the synthesis of lactic acid. Nitrogen is a growth-limiting factor for yogurt starter because milk has an inadequate supply of protein breakdown products to support good growth. Although caseins contain all amino acids necessary for the growth of LAB in milk to high cell density, less than 1% of these are

actually needed (Kunji et al., 1996). Hence, to obtain nitrogen for their growth, these organisms have to rely on their own enzyme systems.

One important result of the addition of the bacteria necessary for fermentation is the resulting proteolytic activity of yogurt bacteria. Protein degradation by microbial enzymes in yogurt is a desirable process that improves milk digestibility and enhances nutritional quality. Lactic acid bacteria are usually weakly proteolytic; however, they do cause a significant degree of proteolysis in yogurt (Abu-Tarboush, 1995).

2.6.2.1.1 The proteolytic system

The proteolytic enzymes found in different species of LAB show different protease activities and complex system of endo- and exopeptidases, which may differ in nature, specificity and cell location (Kunji et al., 1996). In general, LAB possess:

a. Proteases located in the microbial cell envelope that permit the degradation of caseins into oligopeptides
b. Peptide transport systems that allow the internalization of the released oligopeptides
c. Intracellular peptidases that hydrolyse the oligopeptides into peptides or into amino acids to be used by the cells (Kunji et al., 1996; Juillard et al., 1998).

It has been established that degradation of caseins is initiated by a single cell wall-bound extracellular serine-protease. Lactic acid bacteria typically possess only one cell-envelope protease (CEP) but the presence of two CEPs was reported in strains of *L. helveticus* and *L. delbrueckii* ssp. *bulgaricus* (Stefanitsi et al., 1995; Pederson et al., 1999). Cell envelope proteases (PrtP) have a strong preference for hydrophobic caseins. These proteases are critical for growth of LAB in milk because they hydrolyse casein into more than 100 smaller peptide fragments. They have very broad substrate specificity and the biochemical properties of the proteinases of the various LAB are very similar, most of them being serine-proteases of similar size. *Lactococcus* PrtPs are divided into PI- and PIII-type enzymes, distinguished by their substrate specificity for α_{s1}-, β-, and κ-caseins (Kunji et al., 1996). The PI-type primarily degrades β-casein that is cleaved into more than 100 different oligopeptides ranging from 4 to 30 amino acid residues. PI-type enzyme cleaved κ-casein to a lesser extent, whereas the PIII-type is able to cleave α_{s1}-, β-, and κ-caseins equally well. For *Lactobacillus*, CEPs PI-, PIII-, the intermediate PI/PIII-type, and some novel type substrate specificities were reported, whereas CEP exhibiting the intermediate PI/PIII-type specificity was purified from *S. thermophilus* (Savijoki et al., 2006). However, it has been observed that

two strains of dairy lactobacilli showing same proteolysis measurement can markedly differ in their protease specificity (Oberg et al., 2002).

The oligopeptides transported into the cells are further broken down by the action of their peptidases. Apparently, the failure to grow is related to the inability of cells to hydrolyse the peptides transported by the peptide transport system. All the peptidases are located intracellularly and concerted action of peptidases is required for complete degradation of accumulated peptides. The peptidase system of lactococci and lactobacilli are understood to be almost similar, although some strain variations have been reported. The analysis of the genome of *L. acidophilus* NCFM confirmed that the organism encoded for 20 peptidases for protein degradation and peptide utilization along with two complete oligopeptide transport systems (Altermann et al., 2005). However, it is reported that a cell envelope-bound proteinase is rarely present in *S. thermophilus* and that there are two additional peptidases, an oligopeptidase and an amino peptidase, which could be involved in bacterial growth by supplying amino acids and in development of flavour in dairy products (Fernandez-Espla and Rul, 1999). Very little is known about the proteolytic enzyme systems of *Bifidobacterium* species. Analysis of the genome of *B. longum* NCC2705 predicted more than 20 peptidases, including general aminopeptidases, peptidases specific of proline-residues, dipeptidases, and endopeptidases, as well as ABC-type transporter sytems specific for oligopeptides but not the presence of a cell envelope-associated protease. It was observed that aminopeptidase activity of *B. animalis* ssp. *lactis* was higher when grown in milk with no cell-wall bound caseinolytic activity. However, the combined action of intracellular proteolytic enzymes could degrade caseins (Janer et al., 2005). Variations in substrate specificity, aminopeptidase activity and in the enzymatic systems of *L. helveticus* and *L. delbrueckii* ssp. *lactis* and *L. delbrueckii* ssp. *bulgaricus* have also been reported (Gatti et al., 2004). In contrast to the lactococcal proteolytic system, considered as a model, *L. delbrueckii* ssp. *bulgaricus* has shown a high prolyly amino-peptidase activity that offers an additional degradative pathway supplying the cell with essential proline residues (Morel et al., 1999).

The metabolic activity of proteolysis has gained significance in recent years ever since it was realised that it influences the potential release of physiologically active peptides, the bioactive peptides. It is probable that part of the beneficial effects attributed to the foods containing probiotics is associated with bioactive peptides liberated by the proteolytic action of these bacteria (Korhonen and Pihlanto-Leppälä, 2004). Cell-wall bound proteases of LAB, as well as enzymes from endogenous microflora of milk, including digestive enzymes, have

the capacity to release bioactive peptides that are in an inactive state within the sequence of milk proteins. Microbial proteolysis being highly specific, can lead to the release of very potent bioactive peptides. The small peptidases produced by endopeptidases in the bacterial cells may be excreted into the milk product by some sort of exchange of these peptides over the cell membrane (Kunji et al., 1996), or, more likely, as a result of the lysis of the bacterial cell. The intracellular peptidases that escape from their intracellular location due to lysis of bacterial cells may also act on the large oligopeptides produced by the action of the cell-wall proteases and contribute to the pool of bioactive peptides in the fermented milk. In addition to the release of biologically active peptides after fermentation, microbial proteolysis may also expose the inner protein bonds, favouring the action of digestive enzymes and the release of potentially active peptides. The major milk proteins most susceptible to proteolysis, α_{s1}- and β- casein, have 100-209 amino acid residues in their sequence and therefore have the capacity to release more than 20,000 different peptides (Matar et al., 2003).

2.6.2.2 Release of bioactive peptides

It is well known that, apart from nutritional value, proteins also possess biological and physicochemical properties of significance to human health. Research carried out during the last 10-15 years has shown that the caseins and whey proteins can be an important source of biologically active peptides or bioactive peptides. Bioactive peptides are described as ‘food derived components that in addition to their nutritional value exert a physiological effect in the body’ (Vermeirssen et al., 2004).

Bioactive peptides usually contain 3 to 20 amino acid residues per molecule. They have been found to have specific activities, such as antihypertensive, antioxidative, antimicrobial, immunomodulatory, opiod or mineral-binding activities. Many milk-derived bioactive peptides reveal multifunctional properties, i.e., specific peptide sequences may exert two or more different biological activities. Due to their physiological and physicochemical versatility, milk-borne bioactive peptides are regarded as important ingredients for health-promoting functional foods (Korhonen and Pihlanto-Leppälä, 2004). The major means for producing biologically active peptides from milk peptides is shown in Figure 2.2. Of these, the focus of this review is on the peptides regulating the cardiovascular system, more specifically, those that exhibit angiotensin-I converting enzyme (ACE)-inhibitory and hypocholesterolemic effects.

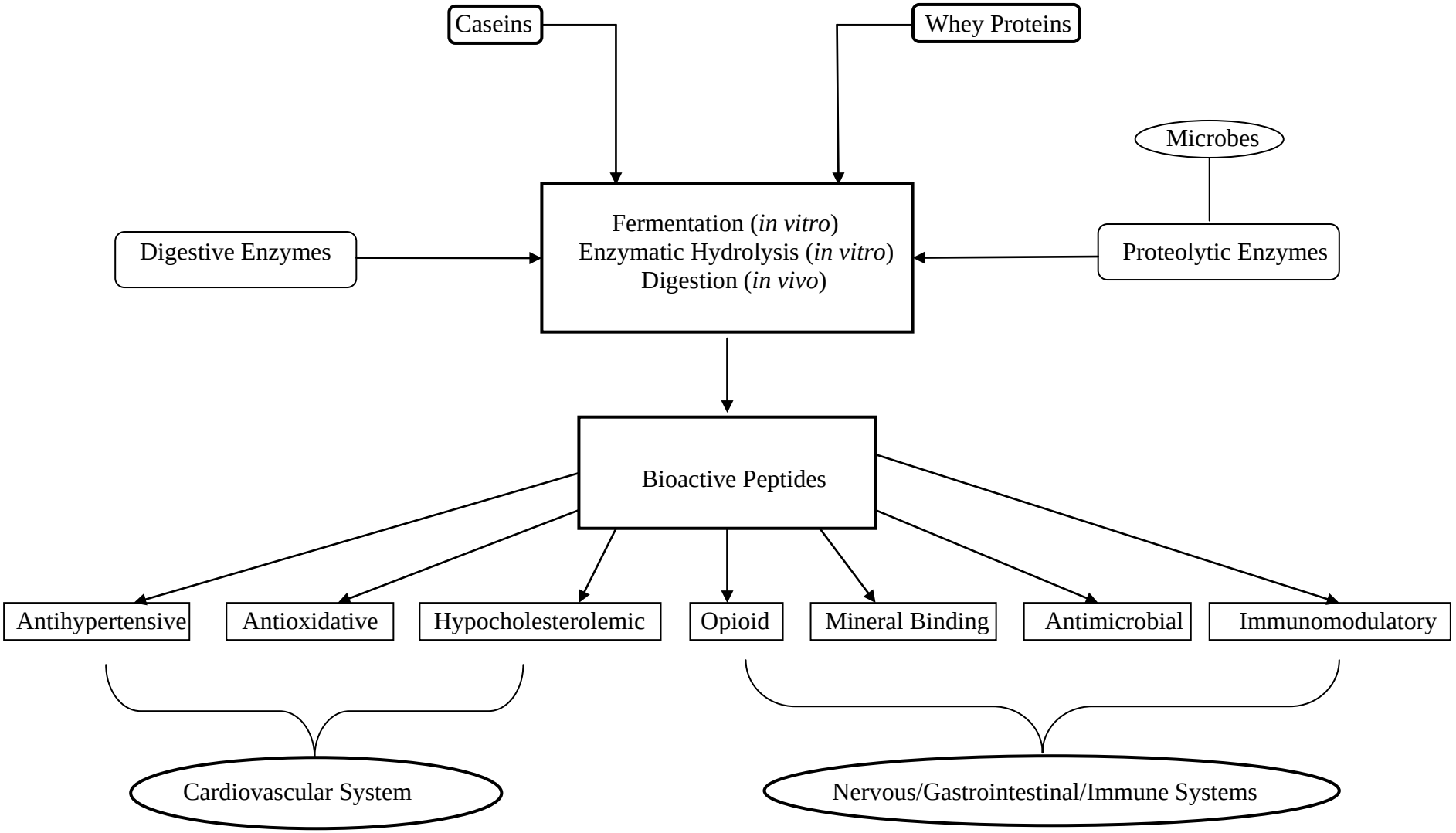

Figure 2.2 Potential means of formation of biologically active peptides from major milk proteins.

2.6.2.2.1 ACE-inhibitory peptides

According to mode of action, antihypertensive peptides are considered to be ACE-inhibitors. Angiotensin-I converting enzyme has been associated with the rennin-angiotensin system, which regulates the peripheral blood pressure. Inhibition of ACE is understood to exert an antihypertensive effect through a decrease of angiotensin II and an increase of bradykinin. Due to the multifunctional property of ACE, it is hypothesised that its inhibition may also have an effect on other regulatory systems involved in immuno-defense and nervous system activities (Fitzgerald and Meisel, 2003).

Angiotensin-I converting enzyme-inhibitors are thought to be competitive substrates for ACE. The primary structural feature governing this inhibitory response is the C-terminal tripeptide sequence. It is proposed that these peptides may interact with subsites s_1, s_1' and s_2' at the active site of ACE (Figure 2.3). It appears that ACE prefers substrates and inhibitors containing hydrophobic amino acid residues in the three C-terminal positions (Cheung et al., 1980). Generally, aliphatic, basic and aromatic residues are preferred in the penultimate positions, while aromatic, proline and aliphatic residues are preferred in the ultimate positions. The positive charge of Arg or the ε-amino group of Lys at the C-terminus has also been shown to contribute to the ACE-inhibitory potential of several peptides (Vermeirssen et al., 2003; Cheung et al., 1980).

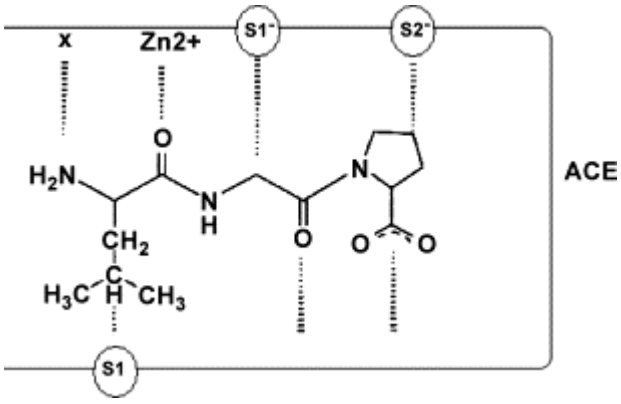

Figure 2.3 Active site of ACE showing the three subsites for interaction.

(Source: Hong et al., 2008)

Lactic acid bacteria have the proteolytic system that hydrolyses milk proteins to release ACE-inhibitory peptides (Yamamoto et al., 1993). ACE-inhibitory and antihypertensive peptides originating from milk usually contain up to 10 amino acids. The majority of milk protein derived ACE-inhibitors have moderate inhibitory potencies, usually

within an IC_{50} range of 100-500 µmol/L (Hayes et al., 2007). Thus, strain selection is one of the main factors that influence the release of ACE-inhibitors in dairy fermentations (Takano, 2002; Korhonen and Pihlanto, 2003). However, peptides with ACE-inhibitory activity may also be formed by *in vitro* hydrolysis of milk proteins using microbial and digestive enzymes (Miguel et al., 2007; Otte et al., 2007; Ortiz-Chao et al., 2009).

Proteolytic strains of the LAB species *L. helveticus, L. casei, L. plantarum, L. rhamnosus, L. acidophilus, L. lactis* ssp. *lactis* and *L. lactis* ssp. *cremoris,* as well as the two species used in traditional yogurt production *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus* have been used to produce fermented milks containing a particularly high number of peptides including ACE-inhibitory and antihypertensive peptides (López-Fandiño et al., 2006). Some ACE-inhibitory peptides are products of extracellular proteases alone such as the large β-casein fragments produced by the extracellular proteases from *L. helveticus* CP790 (Yamamoto et al., 1993) whereas others are most likely the result of the concerted action of both proteases and peptidases such as a dipeptide (YP) isolated from a yogurt like product fermented by *L. helveticus* CPN4 (Yamamoto et al., 1999). Algaron et al. (2004) indicated that the specific peptidase activity of LAB affects the bioactive nature of the peptides produced. Yamamoto et al. (2004) also reported that the gene product of *pepO* gene plays a role in processing the antihypertensive peptides derived from *L. helveticus* fermentation of milk proteins. Ueno et al. (2004) purified and characterized an endopeptidase from *L. helveticus* CM4 and verified that this peptidase can generate the tripeptides IPP and VPP using oligopeptides as substrate.

It has been reported that fermented milk produced by mixing several types of microbes might contain a wider variety of functional substances than milk cultured with a single strain (Kuwabara et al., 1995). Inclusion of probiotics to yogurt has been shown to enhance *in vitro* ACE-inhibitory activity due to improved proteolytic activity (Donkor et al., 2007b). Nielsen et al. (2009) found that the pH at the end of fermentation influences the ACE-inhibitory activity of fermented milk which varies with the strain of LAB used and concluded that proteolysis should not be too extensive. Additionally, LAB also possess a transport system for amino acids, and di-, tri- and oligopeptides. As a result of this system, the residual levels of peptides with bioactivity, such as ACE-inhibitory activity increases in fermented milks. The oligopeptides derived from milk proteins as a result of fermentation can be an additional source for the liberation of bioactive peptides when further degraded by intracellular peptidases after the lysis of bacterial cells. The digestive enzymes in the gastrointestinal tract may also further degrade long oligopeptides leading to possible release

of bioactive peptides. Once liberated in the intestine, bioactive peptides may act locally or pass through the intestinal wall into the blood circulation and end up at a target organ.

Many studies have reported the formation and presence of ACE-inhibitory peptides in fermented milks such as sour milk (Nakamura et al., 1995 a, b), fermented milk treated with pepsin and trypsin (Rokka et al., 1997), quarg (Meisel et al., 1997) and yogurt (Dionysius et al., 2000).

2.6.3 Antihypertensive effect

Hypertension is defined as a sustained increase in blood pressure (BP) and is a controllable risk factor in the development of a number of cardiovascular diseases (CVD) such as stroke and coronary infarction. Uncontrolled high BP increases the risk for CVD, stroke, heart failure and kidney disease. Even a small decrease in BP results in a significant reduction in the risk of CVD and a 5 mm Hg reduction in diastolic BP reduces the risk of heart disease by approximately 16% in hypertensive subjects (Fitzgerald et al., 2004). Such a reduction in diastolic BP corresponds to a decrease in systolic BP by 9-10 mm Hg. It has been estimated that a decrease in systolic BP by 3 mm Hg would reduce the risk of stroke by about 10-13% (Tuomilehto et al., 2004). The high cost of and potential adverse side effects associated with pharmacological therapy for hypertension have encouraged individuals to adopt lifestyle modifications such as weight reduction, low-fat dairy products, dietary sodium reduction and regular physical activity to combat hypertension (Miller et al., 2007b). The blood pressure lowering effects of specific hydrolysates of casein and whey proteins or fermented dairy products provide compelling evidence for a beneficial role of dairy peptides to induce clinically significant reductions in systolic BP and diastolic BP with no reported adverse effects (Huth et al., 2006).

The bioactive peptides such as ACE-inhibitory peptides must reach their target organ intact to exert their effects *in vivo*. Degradation of peptides in the acidic environment of the stomach, alkaline conditions of the small intestines as well as hydrolysis by the brush border peptidases can either activate or deactivate ACE-inhibitory peptides before they reach the portal circulation. Therefore, only those ACE-inhibitors that are not affected by the action of angiotensin-II and gastrointestinal enzymes or those that are converted to stronger ACE-inhibitors exert antihypertensive effects *in vivo* (Korhonen and Pihlanto, 2003; Vermeirssen et al., 2003). Didelot et al. (2006) suggested that in case of oral ingestion, purification of the bioactive peptide would be less suitable than using the whole hydrolysate. The release of bioactive peptides could be reinforced after ingestion of the whole hydrolysate by *in vivo*

digestion, whereas intake of an *in vitro* highly active pure peptide would be lost during this *in vivo* digestion.

Due to the incomplete and often unknown bioavailability of the ACE-inhibitory peptides following oral administration, it is difficult and unreliable to predict the *in vivo* antihypertensive effect based on inhibitory activity *in vitro* (Erdmann et al., 2008). Although valuable information can be obtained from *in vitro* model systems regarding the proteolytic/peptideolytic stability and susceptibility to intracellular passage of these peptides, the actual hypotensive effects can be reliably assessed only through *in vivo* studies (Fitzgerald and Meisel, 2003). The *in vivo* effects are tested in spontaneously hypertensive rats (SHR), which constitute an accepted model for human essential hypertension (López-Fandiño et al., 2006). Numerous rat studies have been performed to determine the hypotensive effect of milk protein derived ACE-inhibitors. The maximal decrease in systolic BP achieved in SHRs using various peptides from milk proteins is summarized in Table 2.12.

In general ACE-inhibitory peptides that have been found to have antihypertensive activity in SHR have IC_{50} values lower than 150 µM. However, in some cases, the extent of ACE-inhibitory activity of the peptide is not correlated with the antihypertensive activity (Matar et al., 2003). Some peptides show strong antihypertensive activity at a low dose even though they possess a low ACE-inhibitory activity (Maeno et al., 1996). Thus, there appears to be no direct relationship between the extent of systolic BP decrease and the IC_{50} values for the different peptides tested to date (Fitzgerald et al., 2004). This implies that apart from ACE-inhibition, milk peptides may exert antihypertensive effect through other mechanisms also, such as inhibition of the release of endothelin-I by endothelial cells (Maes et al., 2004), stimulation of bradykinin activity (Perpetuo et al., 2003), enhancement of endothelium-derived nitric oxide production (Sipola et al., 2002), enhancement of the vasodilatory action of binding to opiate receptors (Nurminen et al., 2000) and a vascular relaxing mechanism (Miguel et al., 2007). Such a view has been supported by the study of Fuglsang et al. (2003) who observed that *in vitro* 8 of 9 potential ACE-inhibitory peptides were competitive inhibitors of ACE but only three of them exhibited very moderate effect *in vivo*.

Table 2.12 Milk-protein derived peptides displaying hypotensive effects in SHR.

Milk protein	Peptide fraction	Maximum decrease in systolic BP (mm Hg)	Reference
α_{s1}-Casein	f(1-9)	-9.3	Saito et al. (2000)
	f(23-24)	-34.0	Karaki et al. (1990)
	f(90-94)	-25.0	del Mar Contreras et al. (2009)
	f(104-109)	-13.0	Maeno et al. (1996)
	f(143-149)	-20	del Mar Contreras et al. (2009)
	f(146-147)	-32.1	Yamamoto et al. (1999)
	f(194-199)	-14.0	Karaki et al. (1990)
α_{s2}-Casein	f(89-95)	-15.0	del Mar Contreras et al. (2009)
	f(189-192)	-5.0	Maeno et al. (1996)
	f(190-197)	-3.0	Maeno et al. (1996)
	f(198-202)	-9.0	Maeno et al. (1996)
β-Casein	f(59-61)	-21.0	Abubakar et al. (1998)
	f(59-64)	-22.0	Abubakar et al. (1998)
	f(60-68)	-7.0	Saito et al. (2000)
	f(74-76)	-28.3	Nakamura et al. (1995a)
	f(80-90)	-8.0	Abubakar et al. (1998)
	f(84-86)	-32.1	Nakamura et al. (1995a)
	f(140-143)	-2.0	Maeno et al. (1996)
	f(169-174)	-32.2	Maeno et al. (1996)
	f(169-175)	-31.5	Maeno et al. (1996)
	f(177-183)	-10.0	Karaki et al. (1990)
α-Lactalbumin	f(50-53)	-23.0	Mullally et al. (1996); Nurminen et al. (2000)
β-Lactoglobulin	f(58-61)	-20.0	Hernández-Ledesma et al. (2007)
	f(78-80)	-31.0	Abubakar et al. (1998)
	f(103-105)	-20.0	Hernández-Ledesma et al. (2007)
Bovine serum albumin	f(221-222)	-27.0	Abubakar et al. (1998)
β_2-Microglobulin	f(18-20)	-26.0	Abubakar et al. (1998)

Most LAB produce ACE-inhibitors during milk fermentation. However, the activity, and thus the *in vivo* potential of the fermented milk, varies with the strain (Fuglsang et al., 2003). Some studies have recorded lowering of blood pressure in animals and human subjects fed fermented milks. Seppo et al. (2002) found that daily consumption of *L. helveticus* LBK-16H fermented milk reduced systolic and diastolic BP by 6.7 and 3.6 mm Hg in hypertensive patients while Tuomilehto et al. (2004) found a 16 - 11 mm Hg decrease in systolic BP but no difference in diastolic BP when subjects with mild hypertension were fed sour milk fermented with *L. helveticus*. Similarly, Chen et al. (2007) found that the whey separated from low-fat milk fermented with five mixed LAB when fed orally to SHR for 8 wks reduced the systolic and diastolic BP by 22 and 21.5 mm Hg, respectively; and Tsai et

al. (2008) observed reduction in diastolic and systolic BP of SHR by 15.9 and 15.6 mm Hg respectively, when fed with whey of milk fermented with *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus*. Costa et al. (2005) reported a reduction in systolic BP of 25 mm Hg when SHR were intraperitoneally administered whey protein hydrolysate.

2.6.4 Hypocholesterolemic effect

Major modifiable risk factors for coronary heart disease include high BP, smoking, and elevated blood cholesterol levels, particularly low-density lipoprotein (LDL)-cholesterol. Other risk factors include diabetes mellitus, physical inactivity, low blood levels of high-density lipoprotein (HDL)-cholesterol, elevated blood triglyceride (TG) levels, and overweight/obesity. Among these, a high blood cholesterol level, particularly LDL-cholesterol, is regarded as one of the major modifiable risk factors. The National Cholesterol Education Program's Adult Treatment Panel III in the USA, classifies desirable total blood cholesterol levels as levels below 200 mg/dL, borderline-high as values between 200 and 239 mg/dL, and high as total cholesterol levels 240 mg/dL and above. Optimal levels of LDL-cholesterol are <100 mg/dL; desirable levels of HDL-cholesterol are 60 mg/dL or higher; and optimal levels of TG are less than 150 mg/dL (Miller et al., 2007a).

Many studies have found a positive correlation between hypercholesterolemia and/or hypertriglyceridemia and the likelihood for developing CVD. Consequentially, treatment for hyperlipidemia-accelerated diseases often includes the improvement of serum lipid distribution through diet modifications. It is generally known that several dietary proteins can improve blood lipid profile (Erdmann et al., 2008).

Hypocholesterolemic effects have been reported for casein- and whey-derived peptides (Nagaoka et al., 2001; Hartmann and Meisel, 2007; Morikawa et al., 2007). Besides, yogurt and other fermented milk products have also been reported to contain some substances that lower serum cholesterol. St-Onge et al. (2000) reviewed the effects and mechanisms of action of fermented dairy products on serum cholesterol concentrations and suggested a moderate cholesterol-lowering action by fermented dairy products. Similarly, the review of animal and human studies by Pereira and Gibson (2002) suggested moderate cholesterol-lowering action by dairy products fermented with appropriate strains of LAB and bifidobacteria. They also reported that prebiotics, FOS, inulin and oligofructose, showed convincing lipid-lowering effects but only at high dose levels (50-200 g/kg) in animals.

Diverse observations have been made by several researchers on the effect of yogurts on the different components of the blood lipid profile of rats. Beena and Prasad (1997)

demonstrated that bifidus yogurt and yogurts fortified with whey proteins can reduce total and LDL-cholesterol of rats but not whole milk; while Yuan and Kitts (1993) observed that feeding yogurt powder diet to normotensive rats reduced plasma TG, total cholesterol and HDL-cholesterol but did not influence their systolic BP. A similar effect was observed on feeding kumiss powder to rats (Ishii and Samejima, 2001). On the other hand, Kawase et al. (2000) observed a substantial increase in HDL-cholesterol level and decrease in TG and atherogenic index in the blood serum of rats fed milk fermented with *L. casei* EMC0409 and *S. thermophilus* TMC1543 along with a decrease in systolic BP of human volunteers consuming the fermented milk. The results of Akalin et al. (1997) suggested that supplementation of the diet of mice with acidophilus yogurt reduced serum cholesterol and LDL-cholesterol without affecting serum TG or HDL-cholesterol. Xiao et al. (2003) reported that as compared to yogurt, *Bifidobacterium* yogurt (*B. longum* BL1) was more effective in improving serum lipids in rats and human volunteers. Nakajima et al. (1992) found that the slime materials produced by *L. lactis* ssp. *cremoris* in ropy fermented milk, vili, had a beneficial effect on rat cholesterol metabolism. A similar observation was made by Maeda et al. (2004) on feeding kefiran, an EPS produced by *L. kefiranofaciens*, along with suppressed increase of BP in SHR rats. Anderson and Gilliland (1999) concluded that regular intake of fermented milk containing *L. acidophilus* L1 by primary hypercholesterolemia patients could help reduce serum cholesterol levels by 3 to 4%, but Kießling et al. (2002) concluded that long-term daily consumption of synbiotic yogurt, containing *S. thermophilus*, *L. lactis*, *L. acidophilus* 145, *B. longum* 913 and 1% oligofructose did not lower the total and LDL-cholesterol in healthy women but increased the serum concentration of HDL-cholesterol that lead to the desired improvement of LDL/HDL ratio.

3.0 Proteolytic Profiles and Angiotensin-I Converting Enzyme and α-Glucosidase Inhibitory Activities of Selected Lactic Acid Bacteria

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3.1 Introduction

Lactic acid bacteria (LAB) are the most common microorganisms found in dairy products and therefore are one of the most extensively studied groups of microorganisms, of which *Lactobacillus*, *Lactococcus*, *Streptococcus* and *Bifidobacterium* genera are most common (Christensen et al., 1999). However, since the discovery of probiosis by Metchnikoff, substantial scientific interest has been directed towards the organisms that provide health benefit. The term 'probiotic', meaning 'for life', originated to describe substances produced by one microorganism which stimulate the growth of others. Among the LAB, *Lactobacillus* and *Bifidobacterium* are the genera considered as probiotics that have a considerable safety record and have been widely exploited by the manufacturers, resulting in a whole range of probiotic fermented milks (Stanton et al., 2003a; Shah 2007).

In the process of milk fermentation, the proteolytic abilities of LAB used as starters plays an important role because it provides these organisms the amino acids essential for their growth (Savijoki et al., 2006). The conversion of peptides to free amino acids and the subsequent utilization of these amino acids is therefore, a central metabolic activity in LAB (Christensen et al., 1999). The utilization of caseins is initiated by cell-envelope proteinase (CEP) which is critical for the growth of LAB in milk because they hydrolyse caseins into more than 100 smaller peptide fragments. The 2nd step in casein utilization includes transportation of peptides generated by CEP into the cell by the action of multiple oligopeptides transport (Opp) systems. After the casein-derived peptides are taken up by the cells, they are degraded by the action of peptidases with differing and partly overlapping specificities (Kunji et al., 1996). Collectively, these enzymes can remove the N-terminal amino acids from a peptide, the specificity depending on the peptide length and the nature of the N-terminal amino acid residue (Kunji et al., 1996; Christensen et al., 1999). Several researchers have observed some differences between the proteolytic systems of LAB (Fernandez-Espla and Rul, 1999; Morel et al., 1999; Lamarque et al., 2001; Letort et al., 2002; Schell et al., 2002; Altermann et al., 2005)

The proteolytic capabilities not only influence the flavour and textural characteristics of the products that contain these LAB, but also generate a host of peptides that are increasingly being associated with health beneficial properties, the bioactive peptides, which has renewed the scientific interest in these capabilities. Proteolysis by naturally occurring

enzymes in milk or by microbial enzymes, especially from LAB, generates bioactive peptides during milk fermentation, thereby enriching the fermented product. Bioactive peptides have been defined as 'specific protein fragments that have a positive impact on body conditions or functions and may ultimately influence health' (Kitts and Weiler, 2003). Once produced, these bioactive peptides may act in the body as regulatory compounds with a hormone-like activity (Gobbetti et al., 2002). Among the bioactive peptides derived from milk proteins, those with blood pressure lowering effect are receiving special attention due to the widespread prevalence of hypertension. Blood pressure regulation is partially dependent on the rennin-angiotensin system. The angiotensin-I converting enzyme (ACE) regulates peripheral blood pressure and its inhibition can exert antihypertensive effects. The technological challenge therefore lies in the manufacture of fermented dairy products with a high concentration of these bioactive peptides. The single most effective way to increase the number of bioactive peptides is to ferment or co-ferment with highly proteolytic strains of LAB (Meisel et al., 1997). However, due to the inherent variations in these capabilities, selection of microorganisms to be used in fermented products, particularly those with specific health claims, is gaining importance. So far a comprehensive study on the enzyme profile, proteolytic activity as well as the ACE-inhibitory activities of yoghurt starter and probiotic organisms has not been conducted. No work has been reported on the α-glucosidase inhibitory activity of LAB.

The current study examines the proteolytic profiles and the angiotensin-I converting enzyme (ACE) and α-glucosidase (α-glu) inhibitory activities of selected strains of yogurt starter and probiotic organisms.

3.2 Materials and Methods

3.2.1 Bacterial strains and their activation

The pure strains of *Streptococcus thermophilus* 1275 and 285, *Lactobacillus casei* 2607 and 15286 and *L. acidophilus* 4461 and 33200 were acquired from the culture collection of Victoria University and *L. delbrueckii* ssp. *bulgaricus* 1092 and 1368 and *Bifidobacterium longum* 5022 were obtained from Australian Starter Culture Research Centre Ltd (Werribee, Vic, Australia). All the strains were activated from their frozen forms (stored in 40% glycerol at -80 °C) by giving one transfer in MRS broth, except *S. thermophilus* which was first transferred in M17 broth. This was followed by 2 successive transfers into 12% sterile reconstituted skim milk (RSM) supplemented with 2% glucose and

1% yeast extract, followed by 2 transfers into sterile RSM. During activation, all organisms were incubated at the optimum temperature of 37 °C, except for *L. delbrueckii* ssp. *bulgaricus* which was incubated at its optimum temperature of 42 °C, for 20 h. The 2nd RSM transfer was used for the conducting the experiments at 37 °C. The rate of inoculation was 1% (v/v) and samples for the various analyses were removed after 0, 3 and 6 h of incubation.

The selection of cultures for further studies was based on the results of this study, particularly the ACE-inhibitory activity. Since these cultures were to be used for yogurt making, the incubation period for which is usually 4 to 5 h, the incubation period selected for this study was 6 h. Although this period of incubation would be considered as short for organisms such as *L. acidphilus, L. casei* and *B. longum*, however, since these organisms were studied along with the faster growing yogurt cultures, it was necessary to maintain a uniform period of incubation for all organisms included in this study.

3.2.2 Growth and pH

The growth and changes in pH were monitored as indicators of growth pattern after 0, 3 and 6 h incubation at 37 °C for all the organisms grown in RSM. The pH was measured using a pH meter (model 8417, Hanna Instruments, Singapore). Since each culture was taken individually for this study, the colony counts were determined using MRS agar for all cultures except *S. thermophilus*, for which M17 agar was used (Shah, 2000a). The plates were prepared by pour plate technique and incubated in anaerobic jars (with the exception of *S. thermophilus*, which was incubated aerobically) at their optimum temperatures, as mentioned above, for 72 h.

3.2.3 Preparation of trichloroacetic acid filtrate

The trichloroacetic acid (TCA) filtrates of samples, removed at 0, 3 and 6 h of incubation, was prepared by mixing 5 mL sample with 1 mL MilliQ water and 10 mL of 0.75 N TCA followed by centrifugation at 4000 × g for 30 min at 4°C. The supernatant thus obtained was passed through a 0.45 µm membrane filter and stored at -20 °C until assayed.

3.2.4 Determination of extent of proteolysis

Proteolytic activity of all cultures was determined by the method of Church et al. (1983). Three milliliters of OPA reagent was added to 150 µL of the TCA filtrate and vortexed for 5 s. Absorbance at 340 nm was measured after 2 min at room temperature, using NovaSpec®-II Spectrophotometer (Pharmacia, England, UK).

3.2.5 Enzyme assays

The aminopeptidase and dipeptidase activities of the extracellular (EE) and intracellular extracts (IE) of all the organisms were determined. The extracts of the organisms grown in MRS broth, except for *S. thermophilus* which was grown in M17 broth, were prepared according to the method of Shihata and Shah (2000). Briefly, after incubation for 18 h, the cells were collected from the growth medium by centrifugation at 12000 × g at 4 ºC for 15 min while the supernatant obtained was used as the EE for enzyme assays. The pellet obtained was washed twice using 0.9% NaCl solution and centrifuged each time to remove the NaCl solution. The washed pellet was dispersed in 1 mL 0.05 M Tris-HCl buffer, pH 8.5, and sonicated for 5 min at 4 ºC. The suspension was then centrifuged at 12000 × g at 4 ºC for 15 min. The supernatant thus obtained was used as the IE for the enzymatic assays. The protein contents of EE and IE were determined as per the method of Bradford (1976).

The aminopeptidase activity of the extracts of all the organisms was determined by the method of Fernandez-Espla et al. (1997) using chromogenic substrates (*para*-nitroanilide-derivatives of L-isomers of Leu, Pro, Lys, Arg, Ala and Met). The assay mixture consisted of 100 µL of the IE, 400 µL of 50 mM Tris HCl buffer (pH 7.0) and 50 µL of 10 mM of each substrate. The mixture was incubated at 37 ºC for 20 min, after which the reaction was terminated by the addition of 1 mL acetic acid (30%, w/v). The concentration of *p*-nitroanilide released was determined by measuring the absorbance at 410 nm using NovaSpec®-II UV-Spectrophotometer. The enzyme activity was calculated considering molar absorption coefficient of *p*-nitroanilide as 9024 $mol^{-1}cm^{-1}$ and expressed as micromoles of substrate hydrolysed per millilitre of extract per min. The specific activity was calculated and expressed per milligram of protein.

The dipeptidase activity of the extracts of all organisms was determined by the method of Wohlrab and Bockelmann (1992) using Ala-Met, Leu-Tyr, Leu-Gly, Ala-His and Pro-Ile as substrates. The reaction mixture contained 10 µL of the IE, 415 µL of 50 mM Tris-HCl buffer (pH 7.5), 50 µL of 22 mM of each substrate solution, 25 µL of peroxidase solution (5 mg/mL of 0.8 M ammonium sulphate solution), 25 µL of L-amino oxidase solution (2 mg/mL deionised water) and 25 µL of 11.5 mM *o*-dianisidine solution. The mixture was incubated at 50 ºC for 20 min followed by the addition of 50 µL of 120 mM dithiothreitol solution to terminate the reaction. The enzyme activity was calculated using a molar absorption coefficient of 8100 $mol^{-1}cm^{-1}$and expressed as micromoles of substrate hydrolysed per millilitre of extract per minute. The specific activity was expressed per milligram of protein.

3.2.6 ACE-inhibitory activity

The ACE-inhibitory activity of the TCA filtrates of all organisms after 3 and 6 h growth at 37 °C was determined by the method of Cushman and Cheung (1971) and Donkor et al. (2005) with some modifications. The method involved the use of 5 mM Hip-His-Leu in 0.1 M borate buffer containing 0.3 M NaCl, pH 8.3, as substrate and rabbit lung ACE (0.1 units/ml). To 200 µL of the substrate solution, 60 µL of the borate buffer and 30 µL of the TCA filtrate were added and pre-incubated at 37 °C for 5 min. This was followed by the addition of 20 µL of the enzyme solution and incubation at 37 °C for 30 min. The reaction was terminated by adding 250 µL of 1 M HCl solution and then mixed with 1.7 mL ethyl acetate. After 10 min of quiescent standing, 1.4 mL of the ethyl acetate layer was siphoned out and dried on a boiling water bath and then in an oven maintained at 80 °C for 30 min. The residual hippuric acid was dissolved in 1 mL of deionized water and absorbance measured at 228 nm using UV/Vis spectrophotometer (Pharmacia, LKB-UltrospecIII). The per cent inhibition was calculated using the following formula:

$$\text{ACE-inhibition (\%)} = \left[1 - \frac{C - D}{A - B}\right] \times 100 \text{ where}$$

A is the absorbance in the presence of ACE and without sample, B is the absorbance without ACE and sample, C is the absorbance with ACE and sample and D is the absorbance with sample but without ACE. The ACE-inhibition was also expressed in terms of IC_{50}, defined as the protein concentration in sample (mg/mL) required to inhibit 50% of the ACE activity. The protein in the TCA filtrates was determined by the method of Lowry et al. (1951) using bovine serum albumin as standard.

3.2.7 α-Glucosidase inhibitory activity

The method of Zhang et al. (2007) was adopted with some modifications to measure the α-glu inhibitory activity of the TCA filtrates of cultures after 3 and 6 h of growth at 37 °C. To 300 µL of 0.1 M phosphate buffer (pH 6.5) 150 µL of 20 mM *p*-nitrophenyl-α-glucoside (SIGMA Chemicals, St Louis, MO, USA) solution and 50 µL of the sample were added and the mixture pre-incubated at 45 °C for 10 min. To this, 100 µL of α-glucosidase enzyme solution (0.2 units/mL, from yeast, SIGMA Chemicals) was added and incubated for another 10 min at 45 °C. The reaction was terminated by adding 2 mL of 0.1 M sodium carbonate solution and the amount of p-nitrophenol released was determined by measuring the absorbance at 400 nm using NovaSpec®-II UV-Spectrophotometer. The percent inhibition and IC_{50} were calculated as indicated for ACE-inhibition.

3.2.8 Statistical analysis

Data were analysed using 1-way analysis of variance at 95% level of significance (Albright et al., 1999). All experiments were replicated twice and all analyses were carried out in triplicates. The results presented are a mean of 6 observations ± standard deviation (SD).

3.3 Results and Discussion

3.3.1 Growth and pH

Changes in growth and pH of all the organisms at 3 hourly intervals during growth in RSM at 37 °C are shown in Figures 3.1 and 3.2, respectively. The variations in initial numbers (0 h) of the different types of organisms is a consequence of the variations in growing ability of these organisms which is also strain specific. All organisms showed increase in log counts after 3 and 6 h of growth (Figure 3.1) with corresponding decrease in pH (Figure 3.2). Among the yogurt cultures, both the strains of *S. thermophilus* showed a similar trend of growth which reflected in a parallel trend of decreasing pH. On the other hand, strain variations in the growth and therefore the decrease in pH was observed for the two strains of *L. delbrueckii* ssp. *bulgaricus*. The growth of *L. delbrueckii* ssp. *bulgaricus* 1368 was slower during the initial 3 hours but improved in the subsequent 3 h of growth, being significantly ($P < 0.05$) higher than strain 1092. A similar pattern was also observed in the decrease in pH of RSM for these 2 strains of *L. delbrueckii* ssp. *bulgaricus*. Donkor et al. (2007a) have also reported slower growth rate for strains of *L. delbrueckii* ssp. *bulgaricus* than that for *S. thermophilus*. Chandan and O'Rell (2006) have indicated that *L. delbrueckii* ssp. *bulgaricus* has poorer ability to breakdown peptides to free amino acids than *S. thermophilus.* This indicates that *L. delbrueckii* ssp. *bulgaricus* are more fastidious than *S. thermophilus* and need simpler sources of nitrogen for their growth. Hence, when grown individually, *L. delbrueckii* ssp. *bulgaricus* takes a longer time to establish in milk as a growing medium that contains complex proteins as a source of nitrogen. Strains of probiotic organisms showed lower growth rates than *S. thermophilus* and therefore less decrease in pH of the medium. Both the strains of *L. casei*, *L. acidophilus* 33200 as well as *B. longum* 5022 showed very little growth during the first 3 h of incubation but showed significantly ($P < 0.05$) improved growth in the subsequent 3 h of incubation. In contrast, the drop in pH was significant ($P < 0.05$) throughout the 6 h of incubation for all the probiotic organisms.

3.3.2 Proteolytic activity

Lactic acid bacteria are fastidious organisms that require an external source of amino acids or peptides that are provided by the most abundant and proline-rich milk proteins, caseins (Savijoki et al., 2006). Thus, proteolytic capabilities of these organisms are important to ensure their growth in milk. The ability of the organisms to break down the milk proteins was determined by measuring the free amino acids generated after 0, 3 and 6 h of growth at 37 °C and is presented in Figure 3.3. All the organisms showed progressive increase in the free amino acids content with time, although the increase for all the organisms was not significant ($P < 0.05$) during the first three hours after which all organisms showed a significant ($P < 0.05$) improvement in their proteolytic capabilities. Donkor et al. (2007a) have also reported variations in the extent of proteolysis by strains of LAB with time, although they observed significant differences only after 12 h of growth. However, Leclerc et al. (2002) have reported a linear increase in the extent of proteolysis with fermentation time for *L. helveticus*. Among the species of LAB studied, *B. longum* surprisingly showed very high proteolytic capability. The ability of *B. longum* to utilise almost all types of substrates (as shown in Tables 3.1 and 3.2) could explain their high proteolytic capability. It is generally accepted that bifidobacteria have poor ability to grow in milk due to their poor proteolytic activity (Dave and Shah, 1998a). Although differences among species of LAB were observed in their ability of utilise milk proteins, no significant ($P < 0.05$) differences were observed in the proteolytic capability among the different strains of any of the organisms, with the exception of those of *S. thermophilus*. These patterns of proteolysis corresponded with the growth patterns of these organisms. Several researchers have reported wide variations in the proteolytic abilities of LAB (Hickey et al., 1983; Oberg et al., 1991).

3.3.3 Aminopeptidase and dipeptidase activity

The specific activity of aminopeptidases and dipeptidases in the extracellular and intracellular extracts of all the organisms is shown in Table 3.1 and 3.2. Variations in the specific activity of aminopeptidases (Table 3.1) were observed for the various species and strains of organism used and also for the various substrates. These differences were significant ($P < 0.05$) between strains of the same species of organism and also between the different species. Different species showed different affinity for the substrates tested, some of which were significantly ($P < 0.05$) different. Both strains of *L. casei* and showed highest aminopeptidase activity (sum of IE and EE) to all substrates except lysine while both strains of *S. thermophilus* showed for lysine. *Lactobacillus acidophilus* 4461 showed highest

activity to proline and alanine containing substrates. Among all the organisms, only the EE of *S. thermophilus* 1275 and the IE of *L. acidophilus* 4461 and *L. delbrueckii* ssp. *bulgaricus* 1092 were able to utilise all the substrates. Similarly, among all the substrates used, only the *para*-nitroanilide derivates of proline, alanine and arginine were hydrolysed by the EE of all the organisms except those of *L. casei*, while the derivatives of proline and lysine were hydrolysed by the IE of all the organisms. Although it is generally accepted that most aminopeptidases and dipeptidases are located intracellularly, presence of some peptidases in the cell wall fraction of *L. delbrueckii* ssp. *bulgaricus* (Gilbert et al., 1994) and presence of extracellular peptidases in the proteolytic pathway of LAB have also been implied (Kunji et al., 1996). Some exceptions were also observed during this study where the EE of some of the organisms showed higher aminopeptidase activity than the IE for some of the substrates used. Both the strains of *S. thermophilus* showed higher EE activity to derivatives of proline, alanine and methionine, while both strains of *L. casei* showed higher EE activity to proline containing substrate only. On the other hand *L. delbrueckii* ssp. *bulgaricus* 1368 showed higher EE activity to alanine, proline and arginine containing substrates while *B. longum* 5022 showed higher EE activity to proline and methionine containing substrates. Shihata and Shah (2000) have also found higher EE activities for aminopeptidase activity of strains of *B. longum* and *L. acidophilus* to arginine.

On the other hand, dipeptidases of both, the EE and IE of all the organisms (Table 3.2), exhibited broad substrate specificity and were able to utilise all the five substrates to varying extents, with the exception of EE of both strains of *S. thermophilus*. Among the species examined, *L. delbrueckii* ssp. *bulgaricus* showed better dipeptidase activity for the substrates tested. The presence of PepX, the peptidase involved in hydrolysing proline containing substrates, has been reported to be present in all species of LAB (Kunji et al., 1996). This ability of proteolytic enzymes to cleave proline containing peptides is an important criterion for organisms used in fermented milk products since caseins are rich in proline (Donkor et al., 2007a). In all the organisms the activity of peptidases in IE was higher than that of EE. *Lactobacillus casei* 2607 showed highest specificity (IE + EE) to Ala-Met and Leu-Tyr, while *L. casei* 15286 exhibited to Ala-Met, *L. delbrueckii* ssp. *bulgaricus* 1092 to Pro-Ile and *L. delbrueckii* ssp. *bulgaricus* 1368 to Leu-Tyr. Kunji et al. (1996) have indicated that the enzymatic pattern of the proteolytic system varies between and within different bacterial species. Variations were observed among the different species. Different species of LAB exhibit different protease activities and complex system of endo

and exopeptidases which differ in nature, specificity and cell location (Kunji et al., 1996; Gatti et al., 2004).

The proteolytic enzyme system in bifidobacteria has not been studied in as great detail as lactococci. El-Soda et al. (1992) have shown the activity of several peptidases in various strains of bifidobacteria. The extracellular and intracellular peptidases of *B. longum* included in this study exhibited broad substrate specificity. This could explain the high proteolytic capability and therefore the high potency of the peptides generated to inhibit ACE. However, among the other LAB, strains that showed higher specific activities of intra- and extracellular aminopeptidases and dipeptidases did not show similar activities, neither in OPA values nor in percent ACE-inhibition. Oberg et al. (2002) have also found that despite similar levels of overall proteolysis, variations in proteinase specificity can be observed. Deutsch et al. (2000) observed wide variations among strains of *L. delbrueckii* ssp. *bulgaricus* in their ability to generate free amino acids. However, strains that showed high intracellular specific activities (Table 3.1 and 3.2) grew better than strains showing lower specific activities of both aminopeptidases and dipeptidases. Also strains of the same species that better utilized proline containing substrates did not show higher ACE-inhibitory activities, except in the case of *L. acidophilus* and *S. thermophilus*.

3.3.4 ACE-inhibition

Fuglsang et al. (2003) and Donkor et al. (2007a) have observed that strains with high proteolytic ability produce peptides that exhibit high ACE-inhibitory potential. The ACE-inhibition potential of all the organisms grown in RSM at 37 °C is presented in Table 3.3. All the LAB had varying abilities to generate peptides showing ACE-inhibitory activity. Gobetti et al. (2000) and Fuglsang et al. (2003) have also observed that several species of LAB produce ACE-inhibitory peptides in milk during fermentation. All the organisms, with the exception of *B. longum*, showed a decrease in ACE-inhibition potential after 6 h of growth as compared to that of 3 h (Table 3.3). These decreases observed in the TCA filtrates of all organisms after 6 h growth were significant ($P < 0.05$), except for *L. acidophilus* 4461, *S. thermophilus* 1275 and *L. delbrueckii* ssp. *bulgaricus* 1092. This indicates that there is a dynamic equilibrium amongst the peptides that show ACE-inhibitory potential which changes due to continued proteolysis. A similar observation has been made by Donkor et al. (2007b). Among the yogurt starter organisms, *L. delbrueckii* ssp. *bulgaricus* 1368 showed highest percent inhibition, while among the probiotic organisms, *B. longum* 5022 showed the maximum ACE-inhibition potential (Table 3.3). The high proteolytic capability of *B. longum*

could have resulted in its ability to generate peptides with greater ACE-inhibitory activity that those generated by lactobacilli. Donkor et al. (2007a) also found that strains of *B. longum* could release peptides having higher ACE-inhibition potential than lactobacilli. Strain variations were also observed in the extent of inhibition and in the extent of decrease in inhibition with time. In general, strains showing higher percent of ACE-inhibition also showed higher proteolysis, except in the case of *L. acidophilus*. Meisel and Bockelmann (1999) have indicated that oligopeptides that cannot be transported into the cell can remain in the medium to exhibit bioactivity.

3.3.5 α-Glucosidase inhibition

Diabetes mellitus is a chronic metabolic disorder due to high blood glucose levels. Controlling postprandial blood glucose is considered critical to early treatment of diabetes. This implies prevention of absorption of carbohydrates after food intake by inhibiting the activity of enteric digestive enzymes such as α-glucosidase (α-glu) (Lebovitz, 2001). The α-glu inhibitory activity of the TCA filtrates of all the organisms is presented in Table 3.4. All organisms exhibited a good α-glu inhibitory activity, being very high (> 80%) in case of *L. casei* 2607, *L. acidophilus* 33200, *L. delbrueckii* ssp. *bulgaricus* 1092 and *B. longum* 5022. The organisms did not show any significant ($P > 0.05$) decrease in the inhibition potential after 6 h of incubation compared to that at 3 h of incubation, with the exception of *L. casei* 2607, *L. delbrueckii* ssp. *bulgaricus* 1368, *B. longum*, and both strains of *S. thermophilus*. It was interesting to note that organisms that showed a high percentage of α-glu inhibition showed lower ACE-inhibitory potential with the exception of *B. longum*. As yet this activity of LAB has not been studied, and it appears that yogurt starter and probiotic organisms may have some anti-diabetic properties.

3.4 Conclusion

The selected strains of *S. thermophilus*, *L. delbrueckii* ssp. *bulgaricus*, *L. casei*, *L. acidophilus* and, *B. longum* in particular, were capable of growing well in milk due to their proteolytic capabilities. When grown individually, *S. thermophilus* showed better growth than *L. delbrueckii* ssp. *bulgaricus* and the probiotics. The increase in growth translated in corresponding decrease in pH. Among the EE of all the organisms, only the EE of *S. thermophilus* 1275 and the IE of *L. acidophilus* 4461 and *L. delbrueckii* ssp. *bulgaricus* 1092 exhibited aminopeptidase activities to all the substrates while the dipeptidases of all organisms exhibited broad substrate specificity and could utilise all the substrates studied. Species and strain specific variations were observed in the activity of their proteolytic enzymes. All the organisms showed varying capabilities to generate bioactive components that could inhibit ACE and α-glucosidase activities *in vitro*. Among the species of LAB studied, *B. longum* showed very high proteolytic capability and ACE-inhibitory potential.

Table 3.1 Specific activity of aminopeptidases in extracellular (EE) and intracellular (IE) extracts of selected lactic acid bacteria.

Lactic acid bacteria		Substrates					
		*p*NA-Leu	*p*NA-Pro	*p*NA-Lys	*p*NA-Arg	*p*NA-Ala	*p*NA-Met
L. casei 2607	EE	ND	0.094 ± 0.002^{aA}	ND	ND	0.018 ± 0.006^{aA}	0.040 ± 0.007^{aA}
	IE	0.107 ± 0.001	0.081 ± 0.005^{aA}	0.125 ± 0.002^{aA}	0.019 ± 0.005^{A}	0.089 ± 0.002^{A}	0.099 ± 0.002^{A}
L. casei 15286	EE	ND	0.109 ± 0.002^{a}	ND	ND	0.035 ± 0.006^{b}	0.053 ± 0.002^{b}
	IE	ND	0.025 ± 0.003^{b}	0.032 ± 0.002^{b}	ND	ND	ND
L. acidophilus 4461	EE	ND	0.111 ± 0.008^{aB}	0.030 ± 0.005^{A}	0.015 ± 0.004^{aA}	0.008 ± 0.004^{aB}	0.083 ± 0.003^{aB}
	IE	0.112 ± 0.004^{aA}	0.251 ± 0.002^{a}	0.142 ± 0.003^{aB}	0.238 ± 0.005^{aB}	0.351 ± 0.004^{B}	0.380 ± 0.005^{aB}
L. acidophilus 33200	EE	ND	0.099 ± 0.003^{a}	ND	0.040 ± 0.002^{bA}	0.020 ± 0.003^{b}	0.052 ± 0.003^{b}
	IE	0.227 ± 0.005^{b}	0.030 ± 0.002^{b}	0.051 ± 0.004^{b}	0.037 ± 0.002^{b}	ND	0.019 ± 0.004^{b}
S. thermophilus 1275	EE	0.028 ± 0.005^{a}	0.137 ± 0.006^{aBC}	0.030 ± 0.003^{aA}	0.040 ± 0.002^{aB}	0.127 ± 0.005^{aBC}	0.102 ± 0.005^{aBC}
	IE	ND	0.085 ± 0.008^{aA}	0.299 ± 0.008^{aB}	ND	ND	0.082 ± 0.010^{A}
S. thermophilus 285	EE	0.014 ± 0.006^{b}	0.104 ± 0.013^{b}	0.023 ± 0.004^{a}	0.048 ± 0.001^{b}	0.090 ± 0.004^{b}	0.085 ± 0.002^{b}
	IE	ND	0.088 ± 0.007^{a}	0.334 ± 0.010^{b}	0.278 ± 0.014^{BC}	ND	ND
L. delbrueckii ssp. *bulgaricus* 1092	EE	ND	0.118 ± 0.006^{aBC}	ND	0.023 ± 0.006^{a}	0.064 ± 0.003^{aB}	0.078 ± 0.007^{aBC}
	IE	0.119 ± 0.002^{aA}	0.155 ± 0.004^{aB}	0.227 ± 0.005^{aB}	0.103 ± 0.002^{BC}	0.073 ± 0.002^{A}	0.162 ± 0.002^{aB}
L. delbrueckii ssp. *bulgaricus* 1368	EE	ND	0.110 ± 0.003^{a}	ND	0.053 ± 0.001^{bA}	0.050 ± 0.006^{b}	0.065 ± 0.010^{b}
	IE	0.012 ± 0.006^{b}	0.084 ± 0.004^{b}	0.121 ± 0.001^{b}	ND	ND	0.114 ± 0.004^{b}
B. longum 5022	EE	0.017 ± 0.006^{A}	0.135 ± 0.003^{BCD}	0.008 ± 0.005^{C}	0.027 ± 0.003^{B}	0.052 ± 0.004^{B}	0.088 ± 0.005^{B}
	IE	ND	0.050 ± 0.003^{A}	0.034 ± 0.003^{C}	0.069 ± 0.002^{B}	ND	0.024 ± 0.002^{BC}

Results presented are means ± SD of 6 observations at 95% level of confidence.
Specific activity is defined as units of enzyme activity per milligram of protein in the extract.
Means in the same column for EE of strains of each species and IE of strains of each species with different lowercase alphabets are significantly different; Means for EE of different species and IE of different species in the same columns with different uppercase alphabets are significantly different
ND = activity not detected
*p*NA = *para*-nitroanilide

Table 3.2 Specific activity of dipeptidases in extracellular (EE) and intracellular (IE) extracts of selected lactic acid bacteria.

Lactic acid bacteria		Substrates				
		Ala-Met	Leu-Tyr	Leu-Gly	Ala-His	Pro-Ile
L. casei 2607	EE	0.572 ± 0.015^{aA}	0.558 ± 0.10^{aA}	0.652 ± 0.015^{aA}	0.706 ± 0.009^{aA}	0.625 ± 0.006^{aA}
	IE	8.805 ± 0.050^{aA}	5.569 ± 0.152^{aA}	2.597 ± 0.068^{aA}	4.651 ± 0.053^{aA}	2.985 ± 0.027^{aA}
L. casei 15286	EE	0.808 ± 0.011^{b}	0.745 ± 0.018^{b}	0.190 ± 0.019^{b}	1.108 ± 0.012^{b}	0.300 ± 0.018^{b}
	IE	5.308 ± 0.049^{b}	2.769 ± 0.013^{b}	4.389 ± 0.043^{b}	2.561 ± 0.046^{b}	3.107 ± 0.068^{b}
L. acidophilus 4461	EE	0.449 ± 0.023^{aB}	0.356 ± 0.006^{aB}	0.233 ± 0.013^{aB}	0.579 ± 0.003^{aB}	0.147 ± 0.009^{aB}
	IE	7.195 ± 0.039^{aB}	2.788 ± 0.046^{aB}	3.148 ± 0.051^{aB}	3.115 ± 0.056^{aB}	1.627 ± 0.052^{aB}
L. acidophilus 33200	EE	0.797 ± 0.010^{b}	1.036 ± 0.017^{b}	0.411 ± 0.012^{b}	0.472 ± 0.026^{b}	0.473 ± 0.026^{b}
	IE	5.884 ± 0.031^{b}	3.799 ± 0.026^{b}	1.549 ± 0.053^{b}	2.390 ± 0.035^{b}	1.011 ± 0.033^{b}
S. thermophilus 1275	EE	ND	ND	ND	0.309 ± 0.030^{a}	0.273 ± 0.051^{a}
	IE	4.341 ± 0.028^{aB}	4.908 ± 0.015^{aC}	1.307 ± 0.031^{aBC}	3.016 ± 0.035^{aBC}	2.804 ± 0.032^{aB}
S. thermophilus 285	EE	ND	ND	ND	0.859 ± 0.008^{b}	0.421 ± 0.028^{bA}
	IE	6.961 ± 0.039^{b}	5.057 ± 0.044^{a}	1.076 ± 0.036^{b}	2.493 ± 0.022^{b}	2.571 ± 0.056^{b}
L. delbrueckii ssp. *bulgaricus* 1092	EE	0.162 ± 0.032^{aBC}	0.471 ± 0.021^{aBC}	0.500 ± 0.031^{aBC}	0.587 ± 0.024^{aB}	0.328 ± 0.020^{aB}
	IE	6.881 ± 0.065^{aB}	4.072 ± 0.087^{aC}	2.441 ± 0.035^{aB}	3.422 ± 0.066^{aB}	3.162 ± 0.020^{aA}
L. delbrueckii ssp. *bulgaricus* 1368	EE	0.132 ± 0.010^{a}	0.345 ± 0.029^{b}	0.478 ± 0.023^{a}	0.579 ± 0.028^{b}	0.584 ± 0.021^{b}
	IE	3.414 ± 0.035^{b}	7.312 ± 0.038^{b}	3.114 ± 0.073^{b}	3.255 ± 0.033^{b}	2.234 ± 0.028^{b}
B. longum 5022	EE	1.089 ± 0.042^{D}	0.213 ± 0.016^{BC}	0.302 ± 0.019^{BCD}	0.753 ± 0.020^{A}	0.232 ± 0.016^{BC}
	IE	3.306 ± 0.025^{BC}	2.622 ± 0.031^{B}	3.272 ± 0.033^{B}	3.113 ± 0.062^{B}	1.967 ± 0.088^{B}

Results presented are means ± SD of 6 observations at 95% level of confidence.
Specific activity is defined as units of enzyme activity per milligram of protein in the extract.
Means in the same column for EE of strains of each species and IE of strains of each species with different lowercase alphabets are significantly different; Means for EE of different species and IE of different species in the same columns with different uppercase alphabets are significantly different
ND = activity not detected

Table 3.3 Changes in percent ACE-inhibition and corresponding IC_{50} values (mg/ml) of TCA filtrates of lactic acid bacteria grown in reconstituted skimmed milk after 3 and 6 h at 37 °C.

Lactic acid bacteria	3 h filtrate		6 h filtrate	
	ACE-inhibition	IC_{50}	ACE-inhibition	IC_{50}
L. casei 2607	22.74 ±3.06[a*]	0.96	13.73 ± 2.83[a*]	1.58
L. casei 15286	33.20 ± 2.24[b*]	0.55	24.99 ± 2.28[b*]	0.73
L. acidophilus 4461	22.15 ± 2.50[a]	0.82	22.26 ± 2.73[a]	0.81
L. acidophilus 33200	14.90 ± 2.00[b*]	1.23	6.46 ± 1.92[b*]	2.83
S. thermophilus 1275	22.98 ± 3.19[a]	0.76	24.96 ± 1.70[a]	0.70
S. thermophilus 285	24.85 ± 3.09[a*]	0.80	14.66 ± 3.58[b*]	1.35
L. delbrueckii ssp. *bulgaricus* 1092	22.95 ± 2.38[a]	0.94	19.76 ± 3.43[a]	1.09
L. delbrueckii ssp. *bulgaricus* 1368	43.26 ± 2.22[b*]	0.48	31.50 ± 5.11[b*]	0.66
B. longum 5022	62.82 ± 2.72[*]	0.38	66.30 ± 2.43[*]	0.35

Results presented are means ± SD of six observations at 95% level of confidence.
Means in the same column of each time period with different lowercase alphabets indicate significant differences between strains of the same species of LAB.
[*]indicates significant differences between means of different time periods in the same row.

Table 3.4 Changes in percent α-glucosidase (α-glu) inhibition and corresponding IC_{50} values (mg/ml) of TCA filtrates of lactic acid bacteria grown in reconstituted skimmed milk after 3 and 6 h at 37 °C.

Lactic acid bacteria	3 h filtrate		6 h filtrate	
	α-Glu inhibition	IC_{50}	α-Glu inhibition	IC_{50}
L. casei 2607	83.18 ± 1.19^{a*}	0.26	80.34 ± 1.73^{a*}	0.27
L. casei 15286	77.20 ± 0.87^{b}	0.24	77.13 ± 0.90^{a}	0.24
L. acidophilus 4461	76.78 ± 1.72^{a}	0.24	76.46 ± 2.11^{a}	0.24
L. acidophilus 33200	82.86 ± 1.42^{b}	0.22	82.78 ± 2.69^{b}	0.22
S. thermophilus 1275	74.32 ± 3.10^{a*}	0.24	65.98 ± 2.30^{a*}	0.26
S. thermophilus 285	66.94 ± 2.68^{b*}	0.30	75.27 ± 4.10^{b*}	0.26
L. delbrueckii ssp. *bulgaricus* 1092	82.92 ± 4.10^{a}	0.26	84.02 ± 2.24^{a}	0.26
L. delbrueckii ssp. *bulgaricus* 1368	67.62 ± 2.12^{b*}	0.31	74.32 ± 1.61^{b*}	0.28
B. longum 5022	85.23 ± 2.73*	0.28	77.93 ± 2.09*	0.30

Results presented are means ± SD of six observations at 95% level of confidence.
Means in the same column of each time period with different lowercase alphabets indicate significant differences between strains of the same species of LAB.
*indicates significant differences between means of different time periods in the same row.

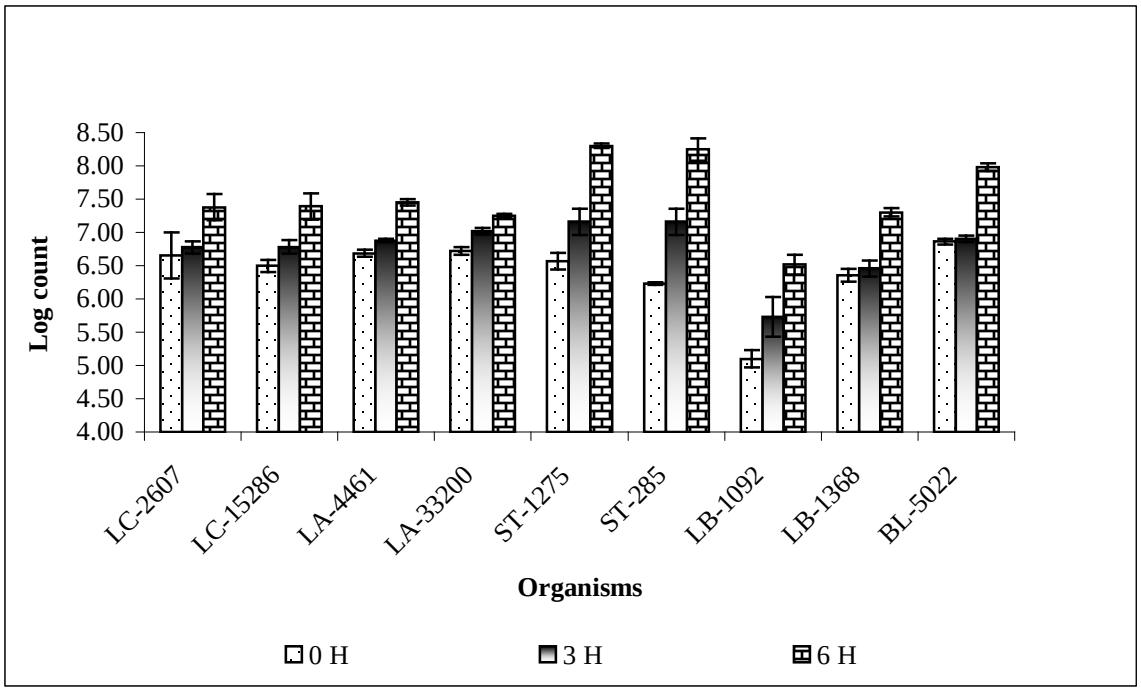

Figure 3.1 Changes in log counts of lactic acid bacteria during 6 h growth in reconstituted skimmed milk at 37 °C. LC = *L. casei* 2607 and 15286, LA = *L. acidophilus* 4461 and 33200, ST = *S. thermophilus* 1275 and 285, LB = *L. delbrueckii* ssp. *bulgaricus* 1092 and 1368, BL = *B. longum* 5022.

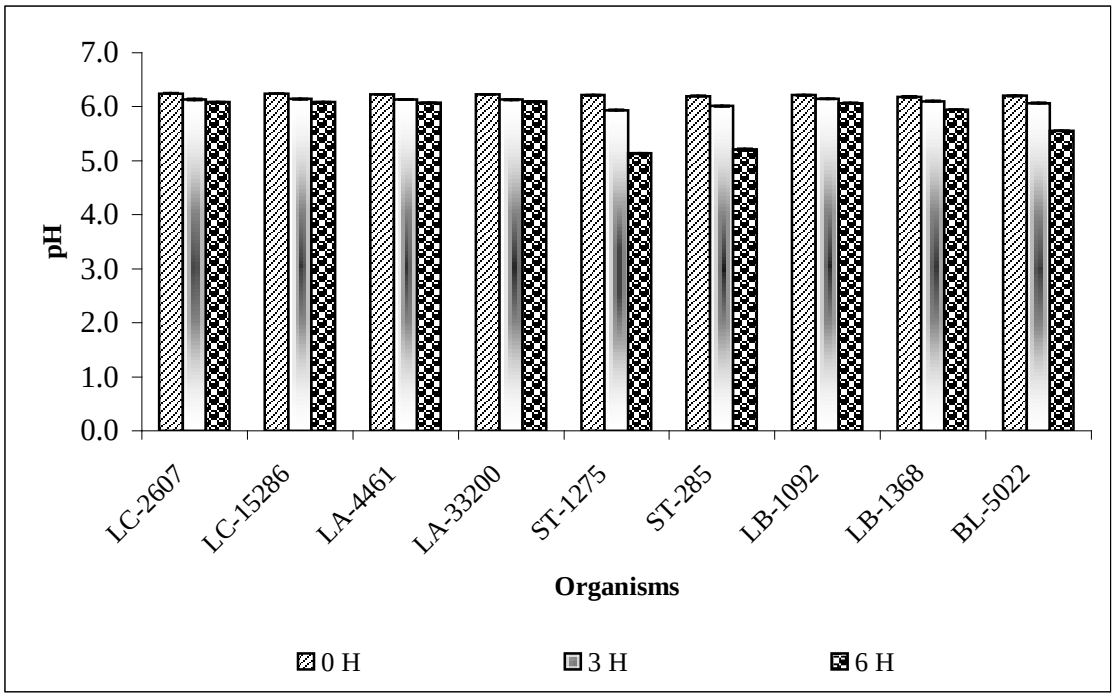

Figure 3.2 Changes in pH of reconstituted skim milk during the growth of lactic acid bacteria at 37 °C for 6 h. LC = *L. casei* 2607 and 15286, LA = *L. acidophilus* 4461 and 33200, ST = *S. thermophilus* 1275 and 285, LB = *L. delbrueckii* ssp. *bulgaricus* 1092 and 1368, BL = *B. longum* 5022.

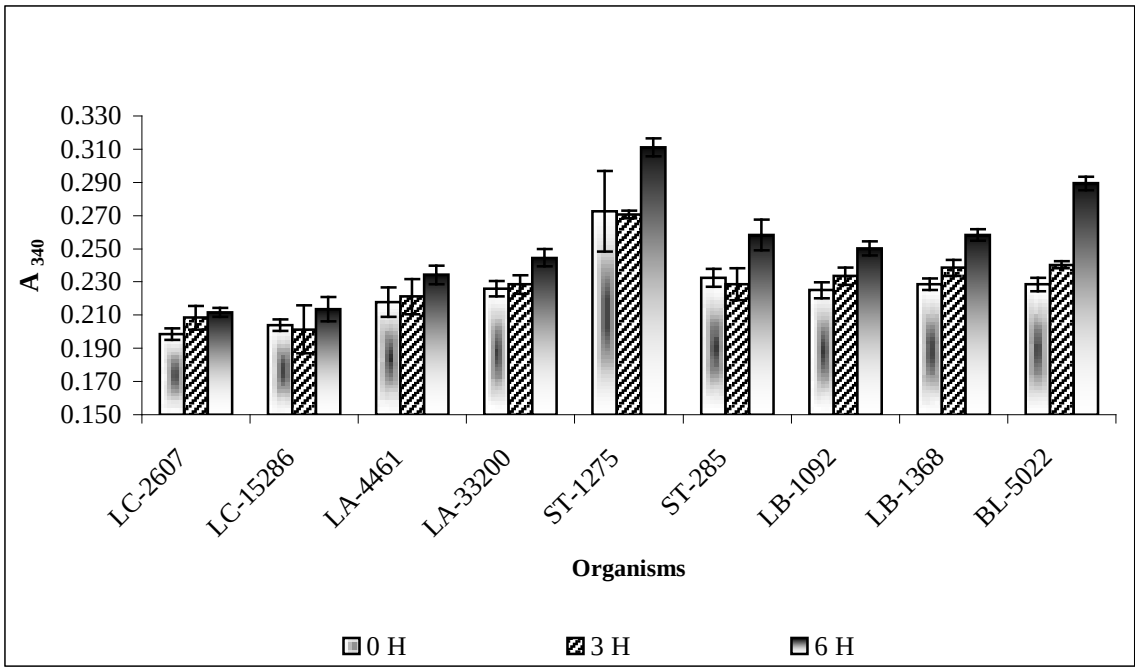

Figure 3.3 Changes in extent of proteolysis (A_{340}) of TCA filtrates prepared from reconstituted skimmed milk fermented by lactic acid bacteria at 37 °C. LC = *L. casei* 2607 and 15286, LA = *L. acidophilus* 4461 and 33200, ST = *S. thermophilus* 1275 and 285, LB = *L. delbrueckii* ssp. *bulgaricus* 1092 and 1368, BL = *B. longum* 5022.

4.0 Effect of Versagel® on the Growth and Metabolic Activities of Selected Lactic Acid Bacteria and on Microbial, Chemical and Physical Properties of Low-fat Yogurt

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4.1 Introduction

The current consumer trend is for healthy foods, specifically for foods that are functional. Often these foods focus on slimming, nutrition, energy and well-being. They are categorized as medicinal/cosmetic, natural or organic foods. With increased awareness of the deleterious effect of milk fat, there has been a boost in the production of low-fat or no-fat foods. Altered products are a class of functional foods in which food products are modified by replacing potentially harmful or undesirable constituents with more beneficial components without affecting product quality (Spence, 2006). Low-fat or no-fat fermented foods containing fat replacers can be placed under this class of functional foods.

Increased awareness about the deleterious effects of consuming excess fat has created a demand for reduced- or no fat-products. Fats in foods contribute to key sensory and physiological properties such as overall flavour, mouth feel, texture, colour and palatability. Reduction or removal of fat therefore creates defects which can be overcome by modifying food formulations. The greatest challenge of formulating low-fat or no-fat products is to deliver the sensory characteristics of the product to the consumer. Among the various approaches to enhance sensory properties is incorporation of fat replacers. The principal objective in developing low-fat or no-fat products is to match the overall product characteristics of a full-fat product. The multifunctional nature of fat in products makes this a difficult task. The role of fat in the physical characteristics and stability of oil-in water emulsion type products, such as yogurt, necessitates the selection of ingredients that can mimic a fat droplet.

Chemically, fat replacers may resemble proteins, carbohydrates or fats, and can be categorized into 2 groups, namely, fat substitutes and fat mimetics. Fat substitutes are macromolecules that physically and chemically resemble triglycerides and can theoretically replace fat in foods on a one-to-one basis. On the other hand, fat mimetics are substances that imitate organoleptic or physical properties of triglycerides but cannot replace fat on a one-to-one basis in foods. Dairy foods use both categories of fat replacers but in fermented foods such as yogurt, fat mimetics are most commonly used (Akoh, 1998).

The introduction of protein-based fat mimetic ingredients in the late 1980s is considered to be a breakthrough in food processing technology which revolutionized the category of reduced fat and fat free foods. The active approach to replacing fats in low-fat or no-fat foods is to add fat mimetic ingredients. These ingredients either physically replace fat or modify the interactions of the remaining components. Another “systems approach” is to

consider the entire food matrix as the system to be manipulated. Proteins are major structural elements of several types of foods, including dairy and meat products. The protein matrix in these products is drastically altered by the removal of fat. In such foods, replacement of fat not only requires building in positive fat like attributes by addition of fat mimetics, but also control the impact of protein interactions in their new fat-free environment that may or may not be detrimental to product quality. Protein-based fat mimetics have proven to be most valuable for fat replacement in oil-in-water emulsion products (Miller, 1994).

Most of the protein-based fat mimetics are in the form of microparticulates. These ingredients play a critical role in a fat-mimetic system by providing the dispersed properties of fat and oil necessary in typical oil-in-water emulsion products. In this role they physically occupy the space previously occupied by emulsified fat droplets (Miller, 1994). The use of protein microparticles has made it possible to retain traditional sensory qualities while substantially reducing the fat content of foods (Singer, 1996). However, it is now being increasingly understood that rheological matching needs to be viewed in the context of its implications for parameters such as physical, chemical and microbiological stability. Since fat reduction can have a definite effect on the physical stability of the product, the fat replacing ingredient should have the ability to maintain physical stability in products such as fat modified yogurts. Among the various replacers available in market, Versagel® is one such whey protein based replacer developed at Food Science Australia (www.foodscience.csiro.au/gelled-food).

Yogurt is defined as ‘a product resulting from milk fermentation with a mixed starter culture consisting of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*’ (Shah, 2003). The recognition of yogurt as a healthy food is a key factor that continues to boost the consumption of yogurt worldwide (Vierhile, 2006). Apart from being nutritionally good, it is now well established that physiologically active peptides are produced from milk proteins during fermentation by lactic acid bacteria (LAB). Among the various bioactive peptides generated by proteolysis of milk proteins during fermentation, the major interest has been on angiotensin-I converting enzyme (ACE) inhibitory peptides. Many industrial cultures are believed to be proteolytic enough to generate bioactive peptides. (Gobbetti et al., 2002, 2004a; Shah, 2004; Korhonen and Pihlanto, 2006; Shah, 2006a). Gobbetti et al. (2000) have reported that milk fermented with *L. delbrueckii* ssp. *bulgaricus* and *L. lactis* ssp. *cremoris* produced peptides showing high ACE-inhibition *in vitro*. Ashar and Chand (2004) have also identified such peptides from milk fermented with

L. delbrueckii ssp. *bulgaricus* and in combination with *S. thermophilus* and *L. lactis* ssp. *lactis* biovar *diacetylactis*.

Yogurt is formed by the slow fermentation of lactose to lactic acid by the thermophilic starter bacteria. Thus, it is a weak viscoelastic acid gel. For milk-based gels, textural attributes can be as important as flavour in determining a consumer's acceptability of the product (Pereira et al., 2006). The overall visual appearance, microstructure and rheological properties of these products are important physical attributes which contribute to the overall sensory perception and functionality of these products (Lucey, 2002). Both casein and whey proteins are involved in the formation of yogurt gels. The texture of an acid milk gel such as yogurt is created from the manner in which the constituent particles interact to form a continuous colloidal network or microstructure, with variations in dimensions and shapes of the droplets, protein strands and pores. For all textural attributes, both solids-not-fat (SNF) and milk fat content play a significant role and variation in these constituents can result in perceivable differences in the texture of the gels (Pereira et al., 2006; Xu et al., 2008).

The fat in yogurt contributes to its overall flavour, velvety mouthfeel, soft smooth texture and palatability. Reduction of fat has been associated with poor texture and syneresis. Traditionally, increasing the level of milk-solids-not-fat by adding skim milk powder or whey ingredients has been adopted to overcome the problem of weak body (Isleten and Karagul-Yuceer, 2006; Sodini et al., 2005). Fat replacers have also been used in such yogurts to overcome the textural defects (Yazici and Akgun, 2004; Sandoval-Castilla et al., 2004). The inclusion of EPS producing strains of starter cultures in the manufacture of yogurts is yet another approach (Amatayakul et al., 2006a).

Although various food additives are now an integral part of the food industry, including fermented foods, very little work has been carried out to understand the influence of these additives on the activity and growth of cultures used in fermented products. Vinderola et al. (2002) have reported the effect of certain flavouring, colouring and sweetening agents on the growth of lactic acid bacteria (LAB) and probiotics. As low-fat or no-fat yogurts are being commercialized, the use of fat replacers in such products is inevitable. Although some work has been undertaken to study the effect of some commercially available protein based fat replacers on sensory and textural characteristics of yogurts (Yazici and Akgun, 2004; Sandoval-Castilla et al., 2004), so far no study has been carried out to examine their influence on the growth and activity of organisms used in such

products. Such studies are important particularly in products that claim specific health benefits to consumers.

Further, the effectivity of protein-based fat replacers in controlling textural parameters is of prime importance in low/no-fat yogurts. Some studies have been conducted on the physical properties of yogurts fortified with milk solids, whey solids or commercial protein-based fat replacers (González-Martínez et al., 2002; Yazici and Akgun, 2004; Sandoval-Castilla et al., 2004; Remeuf et al., 2003; Lucey et al., 1999; Sodini et al., 2005; Isleten and Karagul-Yuceer, 2006). However, there is no comprehensive study on the effect of protein-based fat replacers on the biochemical and physical properties of low-fat yogurt. Therefore, the objectives of this study were i) to determine the effect of Versagel on the growth of selected yogurt starter cultures and probiotics and also to assess the influence on biochemical activities of these organisms, such as proteolytic activity, angiotensin-I converting enzyme (ACE) and α-glucosidase (α-glu) inhibition activities, and organic acid production and ii) to examine the influence of varying levels of Versagel on the growth of *S. thermophilus* 1275 and *L. delbrueckii* ssp. *bulgaricus* 1368, their organic acid producing and proteolytic abilities, and the resulting ACE-inhibitory potentials as well as on some physical properties of the low-fat yogurts, including spontaneous whey separation, firmness and rheology. Considering that addition of Versagel would add to the cost of yogurt produced, it was decided to examine the lowest possible addition required for improvement in biochemical and textural properties of the yogurt, hence both 1 and 2% Versagel were selected for addition

4.2 Materials and Methods

4.2.1 Bacterial strains and their activation

Pure strains of *S. thermophilus* 1275, *L. casei* 15286 and *L. acidophilus* 4461 were acquired from the culture collection of Victoria University and *L. delbrueckii* ssp. *bulgaricus* 1368 and *B. longum* 5022 were obtained from Australian Starter Culture Research Centre Ltd (Werribee, Vic, Australia). All the organisms were activated as described in Section 3.2.1. The strains used for yogurt making were *S. thermophilus* 1275 and *L. delbrueckii* ssp. *bulgaricus* 1368.

4.2.2 Preparation of Versagel added RSM

Versagel was added at the rate of 1 and 2% (w/v) to RSM (12%) and then autoclaved at 121 °C for 15 min. Versagel was obtained from Gelled Foods Australia (East Ringwood, Vic, Australia).

4.2.3 Preparation of low-fat yogurt

Three types of low-fat yogurts were prepared from skimmed milk containing 0.1% fat and 9.3% total solids (Skinny Milk, Parmalat Foods Pty Ltd., Vic) and standardized to 12% total solids with skim milk powder (SMP). The control yogurt (YV0) was prepared from the standardized skim milk while experimental yogurts YV1 and YV2 were prepared from standardized skim milk added with 1% and 2% Versagel (w/v), respectively. Versagel was obtained from Gelled Foods Australia (East Ringwood, Vic, Australia). The SMP and Versagel were added when the milk temperature reached 60 °C. The yogurt mixes were given a heat treatment of 85 °C for 30 min and then cooled in a cold water bath (4 °C) to 45 °C for inoculation. Active cultures of *S. thermophilus* 1275 and *L. delbrueckii* ssp. *bulgaricus* 1368 were each added at the rate of 1% (w/v) to the mixes (average counts were 2×10^6 of *S. thermophilus* and 3×10^6 of *L. delbrueckii* ssp. *delbrueckii* CFU per g in 0 h samples of yogurt mixes) followed by mixing and pouring 50 mL aliquots into polystyrene yogurt cups with lids and incubated at 42 °C until the pH reached 4.5. The yogurts were then cooled by immediately transferring the cups to a refrigerator at 4 °C and then were stored at the same temperature for 28 days. The changes in pH were monitored and population of starter cultures was enumerated, and proteolysis, ACE-inhibitory activity, organic acid production, firmness and whey separation were determined at day 1, 7, 14 and 28 of storage at 4 °C while rheological parameters were measured at day 1 only. The day 1 analysis was carried out after storing samples at 4 °C for 18 h.

4.2.4 Monitoring growth

The growth at 37 °C was observed for all organisms in RSM as well as in RSM containing 1% Versagel (RSMV1) and 2% Versagel (RSMV2) as described in Section 3.2.2. The results were calculated as $\log_{10}$ colony forming units (CFU) per millilitre of sample and expressed as increase in counts calculated by subtracting log counts at 0 h from those at 6 h.

The growth and viability of the starter cultures, *S. thermophilus* 1275 and *L. delbrueckii* ssp. *bulgaricus* 1368, in the fresh and stored low-fat yogurts was determined by pour plate technique using M17 agar and MRS agar, respectively (Dave and Shah, 1996). The counts were expressed as CFU per gram of yogurt sample.

4.2.5 Measurement of pH

The pH of all RSM samples was measured as indicated in Section 3.2.2. Drop in pH was the difference between the two observations at 0 h and 6 h.

Changes in pH of the 3 types of low-fat yogurt were monitored during storage at 4 °C for 28 days using a pH meter (model 8417, Hanna Instruments, Singapore). The drop in pH during preparation of the yogurts was also monitored by measuring the pH of the samples at the start of incubation (0 h) and then at regular intervals until the pH reached ~ 4.5.

4.2.6 Preparation of filtrates for biochemical analyses

The trichloroacetic acid (TCA) filtrates of RSM samples, removed at 0 and 6 h of incubation, was prepared and stored as described in Section 3.2.3.

The filtrates of fresh (day 1) and stored (days 7, 14 and 28) low-fat yogurt samples were prepared by centrifuging at 4000 × g for 30 min at 4 °C. The filtrates of unfermented samples (0 h) were prepared by lowering their pH to 4.5 with glacial acetic acid followed by centrifugation at 4000 × g for 30 min at 4 °C. All the supernatants thus obtained were filtered through 0.45 µm membrane filter and stored at -20 °C until assayed.

4.2.7 Measurement of proteolysis

Proteolytic activity of all organisms was assessed as described in Section 3.2.4. The increase in free amino acids content was calculated by subtracting the absorbance at 0 h from that at 6 h.

Similarly, the readings of the 0 h samples as well as the reagent blank were deducted from the corresponding readings of fresh and stored yogurt samples to obtain the amount of free amino acids released as a consequence of the proteolytic activity of the starter cultures.

4.2.8 Determination of ACE-inhibitory activity

The ACE-inhibitory activity as well as the protein content of the TCA filtrates of cultures after 6 h growth at 37 °C was determined according to the procedure described in Section 3.2.6.

The ACE-inhibitory activity was determined in the filtrates of freshly inoculated mixes (0 h) as well as of stored samples of yogurt as described in Section 3.2.6. The nitrogen content of the filtrates was determined by the traditional Kjeldahl method and the protein content was calculated by multiplying the nitrogen content with 6.38 (AOAC, 2005).

4.2.9 Estimation of α-glucosidase inhibitory activity

The α-glucosidase (α-glu) inhibitory activity of the TCA filtrates of organisms after 6 h of growth at 37 °C was measured by the procedure described in Section 3.2.7.

4.2.10 Determination of organic acids

The acetic and lactic acids content in the samples incubated at 37 °C for 0 and 6 h as well as in filtrates of freshly inoculated mixes samples (0 h) and stored yogurt samples was determined by the method of Scalabrini et al. (1998) with some modifications. To 1 mL of the sample, 40 µL of concentrated nitric acid and 500 µL of 0.005 M sulphuric acid were added and mixed. The mixture was centrifuged for 30 min at 14000 × g in Eppendorf 5415C centrifuge (Crown Scientific, Melbourne, Vic, Australia). The supernatant was filtered through 0.45 µm membrane filter into high performance liquid chromatography (HPLC) vials for analysis of organic acids. The organic acids were separated using a Varian HPLC (Varian Analytical Instruments, Walnut Creek, CA, USA) fitted with Aminex HPX-87H, 300 x 7.8 mm ion exchange column (Biorad Life Science Group, Hercules, CA, USA) and a guard column maintained at 65 °C. The organic acids were detected using UV/Vis detector at 220 nm. An aliquot of 25 µL of sample was injected into the column and eluted using 0.005 M sulphuric acid as a mobile phase at a flow rate of 0.6 mL/min. The retention times of the samples were compared with those of standard working solutions of L (+) lactic acid prepared from a stock solution of 5.1990 g/50 mL and of acetic acid prepared from a stock solution of 5.0244 g/50 mL. The standard curves of both lactic and acetic acids, obtained from working solutions prepared by diluting 1, 2, 3, 4 and 5 mL of the stock solution to 50 mL with the mobile phase, were used for quantification of the organic acids in the samples.

4.2.11 Measurement of spontaneous whey separation

The spontaneous whey separation in fresh and stored low-fat yogurt samples was measured by the siphon method described by Amatayakul et al. (2006a). Briefly, a cup of yogurt was weighed, immediately on removal from the refrigerator, and tilted at an angle of 45° to collect the surface whey. The collected whey was siphoned out with a syringe to which a needle was attached. The siphoning was performed within 10 s to avoid leakage of whey from the curd. Thereafter, the cups were weighed and whey separation calculated by dividing the weight of whey siphoned with the initial weight of yogurt sample. The results were expressed as percentage spontaneous whey separation.

4.2.12 Determination of firmness of yogurt gels

The firmness of the fresh and stored low-fat yogurts was determined using a texture analyzer TA-XT.2 (Stable Micro Systems, Godalming, UK) with a P20 probe (dia 20 mm) and 25 kg load cell. The speed of penetration was set at 1 mm/s and depth of penetration was

10 mm. The ratio of cup diameter to probe diameter was 3.5:1 (Amatayakul et al., 2006a). The gel strength was expressed in g, indicative of the force required to break the gel. The measurements were performed as soon as the samples were removed from the refrigerator.

4.2.13 Rheological measurements

The viscoelastic properties of the fresh low-fat yogurt samples were determined by small amplitude oscillatory measurement (SAOM) using a controlled stress/controlled rate rheometer (Physica MCR 301, Anton Paar, GmbH, Germany). The rheometer was equipped with a temperature and moisture regulating hood and cone-plate geometry (CP50-1, 50 mm dia, 1° angle and 0.02 mm gap, Anton Paar). The temperature of the system was regulated by a viscotherm VT2 circulating bath and controlled at 5 ± 1 °C with a Peltier system (Anton Paar). The data of the rheological measurements were analysed with the supporting software Rheoplus/32 V2.81 (Anton Paar). All the samples were gently stirred with a plastic spoon prior to loading a portion of the sample on the inset plate. The samples were presheared at a shear rate of 500 s^{-1} for 30 s and the structure allowed to rebuild for 150 s before the SAOM was performed. The samples were subjected to a frequency sweep test using a frequency ramp from 0.1 to 10 Hz at a constant strain of 5% to ascertain the viscoelastic properties. The shear rate, storage modulus, loss modulus and damping factors were recorded for all the samples. The viscosity behaviour of the samples was determined by using the Power equation (Ostwald-de Wael model) and Herschel Bulkley model. The Power equation is

$$\sigma = k \cdot \dot{\gamma}^{n}$$

where σ is the shear stress, k is the consistency index, $\dot{\gamma}$ is the shear rate and n is a dimensionless number that indicates the closeness to Newtonian flow (n <1 indicates pseudoplastic liquid). The Herschel-Bulkley model is

$$\sigma = \sigma_0 + k \cdot \dot{\gamma}^{n}$$

where σ_0 is the yield stress. The larger the value of k, the thicker the product and therefore, the more viscous the fluid (Bourne, 2002).

4.2.14 Statistical analysis

Statistical analyses of data for RSM fermentation studies were performed as described in Section 3.2.8.

The results of studies for low-fat yogurts were analyzed as a randomized block design, split plot in time with treatments and replications as the main plot and time as the sub plot. The data from the rheological models were analysed with one-way ANOVA and Tukey's test for multicomparison of the means. All results were analysed using a general linear model procedure of the SAS system (SAS, 1996) at 95% level of confidence; where required, the correlational analysis between parameters was performed using MS Excel StatPro. The study was replicated three times with two sub sampling (n = 6).

4.3 Results and Discussion

4.3.1 Growth of organisms

The changes in growth of the selected organisms during 6 h at 37 °C in RSM with varying levels of Versagel and RSM are presented in Table 4.1. Among all the organisms studied, *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* grew best while the probiotics showed lower growth in RSM. *Lactobacillus acidophilus* and *B. longum* are known to grow poorly in milk (Shah, 2000a; El-Zahar et al., 2004). Of the yogurt starters, *S. thermophilus* exhibited significant ($P < 0.05$) improvement in growth while *L. delbrueckii* ssp. *bulgaricus* showed a significant ($P < 0.05$) decrease in growth in RSM containing 1% Versagel as compared to RSM alone. However, the increase in counts of *S. thermophilus* as well as the decrease in counts of *L. delbrueckii* ssp. *bulgaricus* in RSMV2 were not significant ($P > 0.05$) as compared to RSM, indicating that the level of Versagel had varying influence on the growth of the yogurt starters. The probiotics also showed varying levels of growth. *B. longum* showed slight, but nonsignificant, progressive increase in growth in RSMV1 and RSMV2. The growth of *L. casei* was slightly improved in RSMV1 but decreased in RSMV2 while growth of *L. acidophilus* was significantly ($P < 0.05$) inhibited at both levels of Versagel. It is possible that because Versagel functions to form good gels, some protein cross linking might have occurred in the sterilized RSM containing Versagel, in particular RSMV2, which in turn may have slowed the growth of the organisms. Ozer et al. (2007) have reported that cross linking of milk proteins by microbial transglutaminase had a growth-slowing effect on yogurt starter bacteria. However, the effect of cross linking appears to vary with the type of organism. Thus, inclusion of Versagel improved the growth of *S. thermophilus* and to some extent of *B. longum* but inhibited that of *L. delbrueckii* ssp. *bulgaricus*, *L. acidophilus* and *L. casei*.

Table 4.2 shows the growth and viability of the starter cultures, *S. thermophilus* 1275 and *L. delbrueckii* ssp. *bulgaricus* 1368, in the 3 types of low-fat yogurts stored at 4 °C. There was an increase of almost 2 log cycles in the counts of the starter cultures at the end of fermentation in all the yogurts regardless of the presence of Versagel (data not shown). The counts of *S. thermophilus* were similar in YV2, YV1 and YV0. Surprisingly, it was observed that the deleterious effect of Versagel on the growth of *L. delbrueckii* ssp. *bulgaricus* when grown individually in RSM (Section 4.4) was not observed in yogurt. This could be due to the effect of co-culturing of *L. delbrueckii* ssp. *bulgaricus* along with *S. thermophilus* during yogurt making. Dave and Shah (1998a) have reported that whey proteins stimulate the growth of *S. thermophilus*. The addition of whey protein-based fat replacer Versagel improved the survival of *S. thermophilus* during storage of yogurt at 4 °C. There was no significant change in the count of *S. thermophilus* in the control yogurts (YV0) during storage. In contrast, *S. thermophilus* showed a significant ($P < 0.05$) increase in counts in YV1 during the 1st week of storage while in YV2 there was a significant ($P < 0.05$) increase in counts during the 2nd week of storage. However, in case of *L. delbrueckii* ssp. *bulgaricus*, there were no significant changes throughout the storage period except the end of storage (day 28) when all the 3 types of yogurts showed a significant ($P < 0.05$) decrease in numbers. Amatayakul et al. (2006a) made similar observation related to viability of *L. delbrueckii* ssp. *bulgaricus* during storage of yogurts and similar results have been reported for *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* by Donkor et al. (2006). In general, starters used for preparation of yogurt are reported to remain viable throughout the storage period of the product (Hamann and Marth, 1984). So far there are no reports on the effect of protein-based fat replacers on the growth and viability of starter cultures in yogurt.

4.3.2 Changes in pH

The drop in pH of RSM during 6 hours growth of the selected organisms at 37 °C is shown in Table 4.1. Yogurt starters, *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus*, showed maximum drop in pH when grown in RSM. Addition of Versagel resulted in a significantly ($P < 0.05$) higher decrease in pH by *S. thermophilus* but not by *L. delbrueckii* ssp. *bulgaricus*. The drop in pH observed for probiotics was much less than that for the yogurt starters in RSM. Further, the addition of Versagel did not result in any significant changes in the pH. These changes can be related to the observed growth pattern of these organisms. Although the log count of *L. acidophilus* decreased in the presence of Versagel, the drop in pH was not significantly affected. Similarly, the slight increase in counts of

B. longum in the presence of Versagel did not translate into greater drop in pH. In concurrence with the growth pattern, addition of Versagel resulted in substantial decrease in pH in case of *S. thermophilus* only.

The pattern of changes in pH during fermentation of the three types of low-fat yogurts YV0 (control), YV1 and YV2 is given in Table 4.3. The pH of skim milk dropped to around 5.80 during the first 3 h of incubation, irrespective of the presence of Versagel. Thereafter, the pH of milk containing 2% Versagel (YV2) dropped steeply in the next 30 min of incubation and then at a slower rate for the remaining period of incubation as compared to milk containing 1% Versagel (YV1) and the control (YV0). However, in the later part of fermentation, the pH of both YV1 and YV2 was almost parallel and both batches of yogurt reached the end point of preparation, pH 4.50, in about the same time compared to the control yogurt which took longer to reach pH 4.50. Overall, yogurts containing Versagel took 20 min less fermentation time than that of the control yogurt. This can, in part, be attributed to the ability of Versagel, which is a modified whey protein, to reduce the buffering capacity of milk and can also be attributed partly to the modified kinetics of bacterial growth, particularly *S. thermophilus*, in the presence of Versagel. Amatayakul et al. (2006a) also observed that increasing the concentration of whey proteins reduced the fermentation time in yogurt. It was also observed that the addition of Versagel caused a drop in pH of the skim milk prior to fermentation (Table 4.3). Yazici and Akgun (2004) have also reported a lowering in pH due to addition of protein-based fat replacers.

The changes in pH in stored samples of the 3 types of low-fat yogurts are given in Table 4.4. The pH of all the 3 types of the fresh yogurts (day 1) is slightly different than the final pH observed at the end of fermentation (Table 4.3). This is due to the equilibration time given during the storage of the freshly fermented yogurts at 4 °C for 18 h as well as the higher temperature of the freshly fermented samples as compared to the stored samples. Moreover, the pH of yogurts containing Versagel was higher than the control yogurts. All the 3 types of yogurt showed a significant ($P < 0.05$) drop in pH during the 1st week of storage. Thereafter, the change (0.02 to 0.05 pH units) was not significant for YV0 and YV1 but the drop (0.1 pH unit) was significant ($P < 0.05$) for YV2 for the 2nd week also, after which there was no change in its pH. All the samples reached a similar pH (4.25-4.29) towards the end of storage. The decrease in pH was in line with an increase observed in the number of *S. thermophilus* (Table 4.2). Donkor et al. (2006) have also reported that changes in pH of yogurts prepared from RSM were not significant when stored at 4 °C.

4.3.3 Changes in proteolysis

The extent of proteolysis in RSM, as measured by absorbance at 340 nm by free amino acids, is given in Table 4.1. Proteolysis was maximal for *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* grown in RSM indicating the proteolytic capabilities of both these organisms and their ability to utilize casein (Christensen et al., 1999). Casein is the major source of free amino acids for LAB to grow in milk; this is dependant on the proteolytic capabilities of these organisms (Savijoki et al., 2006). However, addition of Versagel significantly ($P < 0.05$) reduced the amount of free amino acids generated by *S. thermophilus* despite having improved growth. On the other hand *L. delbrueckii* ssp. *bulgaricus* did not generate any free amino acid in the presence of Versagel (Table 4.1). The ability of *L. delbrueckii* ssp. *bulgaricus* to breakdown peptides to free amino acids is less than that for *S. thermophilus* (Chandan and O'Rell, 2006) and this ability seems to have been further reduced in the presence of Versagel. This could also explain the decreased growth of *L. delbrueckii* ssp. *bulgaricus* in the presence of Versagel. In case of the probiotic organisms, the changes in proteolytic capability followed their growth pattern in RSM, RSMV1 and RSMV2 with *L. casei* showing highest and *B. longum* the lowest activity in RSM. Strains of *L. casei* have been reported to be highly proteolytic (Brandsaeter and Nelson, 1956). Shihata and Shah (2000) also found higher proteolytic activity for strains of *S. thermophilus*, *L. delbrueckii* ssp. *bulgaricus* and *L. acidophilus* than *B. longum*. In all cases 2% Versagel containing medium deterred the proteolytic capabilities, being significant ($P < 0.05$) in case of *S. thermophilus* and *L. casei*, with the exception of *B. longum*. The addition of Versagel to RSM followed by sterilization may have resulted in enhanced protein cross-linking interaction (Lucey, 2004) which might have reduced the availability of the milk proteins for utilization by the organisms. It was observed during the experiment, that RSM with 3% added Versagel gelled during autoclaving. However, the concentration of added Versagel appears to enhance the proteolytic capability of *B. longum* which showed a significant ($P < 0.05$) improvement in RSMV2 compared to RSMV1. Fuglsang et al. (2003) have shown that the amount of free amino groups formed in the medium during fermentation depends on the strain used for fermentation. It appears that Versagel somehow inhibited extensive proteolysis by the selected strains of organisms with the exception of *B. longum*. It is not clear whether these observations are due to some changes in the activities of related enzyme systems of these organisms or due to inherent differences in the proteolytic system of bifidobacteria compared to lactobacilli and streotococci..

The changes in the extent of proteolysis as measured by the amount of free amino acids (Δ absorbance at 340 nm) in the fresh and stored samples of 3 types of low-fat yogurt is shown in Table 4.5. The starter cultures showed higher ($P < 0.05$) proteolytic capability in fresh yogurts containing 2% Versagel (0.487) as compared to those containing 1% Versagel (0.336) and the control (0.386). There was a significant ($P < 0.05$) improvement in this ability during the 1st week of storage in both YV2 and YV1, but the increase was not significant in YV0. All yogurts showed a high increase in the amount of free amino acids towards the end of the storage period (day 28) at 4 °C. These increases were significant in YV0, YV2 ($P < 0.0001$) and in YV1 ($P < 0.05$). Also, the extent of proteolysis in YV0 was higher ($P < 0.05$) than YV2 which in turn was higher ($P < 0.05$) than YV1 on day 28. Addition of Versagel has been reported to suppress the proteolytic capability of *S. thermophilus* 1275 and *L. delbrueckii* ssp. *bulgaricus* 1368 when grown individually in RSM (Ramchandran and Shah, 2008a).

4.3.4 ACE-inhibitory activity

The percentage ACE-inhibitory activity of the TCA filtrates of organisms grown for 6 h at 37°C along with their IC_{50} values are presented in Table 4.6. All the organisms were able to generate bioactive peptides which showed *in vitro* ACE-inhibition to varying extent. *Bifidobacterium longum* and *L. delbrueckii* ssp. *bulgaricus* showed maximum inhibition of ACE when grown in RSM while *L. casei* showed the least. Fuglsang et al. (2003) have reported that production of ACE-inhibitors is not restricted to any single species of bacteria. Fermented milks prepared using proteolytic strains of LAB are known to produce ACE-inhibitory peptides (Smacchi and Gobbetti, 2000; López-Fandiño et al., 2006). Gobbetti et al. (2000) have indicated that several strong ACE-inhibitory peptides can be derived from the milk proteins by fermenting with *L. delbrueckii* ssp. *bulgaricus*. Good correlations could be found between OPA index (OPA value multiplied by 1000) and percent ACE-inhibition exhibited by *S. thermophilus, L. delbrueckii* ssp. *bulgaricus* and *B. longum* ($r = 0.80$, 0.70 and 0.94 respectively) but not for *L. acidophilus* and *L. casei* grown in RSM. Fuglsang et al. (2003) found a strong correlation between OPA index and *in vitro* ACE-inhibition only for strains of *L. helveticus*. The effect of Versagel on percent ACE-inhibition was found to vary with the type of organisms and the levels of addition. A significant ($P < 0.05$) increase in the inhibitory activity was observed when *L. casei* was grown in RSMV1 and RSMV2 while *S. thermophilus, L. delbrueckii* ssp. *bulgaricus* and *B. longum* showed a significant ($P < 0.05$) decrease in RSMV1 and a significant ($P < 0.05$) increase in RSMV2.

Lactobacillus acidophilus was able to generate peptides exhibiting inhibitory activity to the same extent in RSMV1 as in RSM but showed significant ($P < 0.05$) increase in activity in RSMV2. No definite correlation could be observed between OPA index and ACE-inhibition for any of the organisms grown in the presence of Versagel. The results indicate that the concentration of Versagel could influence the ability of LAB to generate potentially active ACE inhibitory peptides being better at 2% level. The corresponding IC_{50} values were quite low suggesting good ACE-inhibitory potential of the peptides generated, but not as low as reported for some organisms in fermented milks (Gobbetti et al., 2000; Gobbetti et al., 2002). However, assuming that these strains may be used in combination in fermented products, better inhibitory potential can be expected. There is no published literature that studied the influence of additives such as fat replacers on the ability of LAB to generate ACE-inhibitory peptides.

The ACE-inhibitory activity (%) and the corresponding IC_{50} (mg/mL) values of the three types of low fat yogurts is shown in Figure 4.1. No ACE-inhibitory activity was detected in the 0 h samples of all the three types of yogurt mixes. Among the three types of fresh yogurts, YV0 had the highest ACE-inhibition (38.17%) followed by YV2 (31.62%) and YV1 (23.95%), the differences being significant ($P<0.05$). The IC_{50} values (mg/mL) also followed the same trend, YV0 (5.22) >YV2 (7.49) >YV1 (10.36). During storage at 4 °C, while the ACE-inhibition (%) of YV0 showed a significant increase after the 1st week and towards the end of storage, YV2 showed a significant decrease during the 1st week followed by an increase ($P < 0.05$) during the 2nd week of storage with a decrease ($P > 0.05$) at the end of the storage period. On the other hand, YV1 exhibited a significant increase ($P < 0.05$) in the ACE-inhibitory activity during the 1st week of storage followed by a decrease ($P < 0.05$) during the 2nd week and finally an increase towards the end of storage at 4 °C. The ACE-inhibition (%) and IC_{50} (mg/mL) values of YV0 remained higher than YV2 followed by YV1 throughout the period of study, all differences being significant ($P < 0.05$) at each period of storage. These differences indicate that addition of Versagel suppressed the generation of ACE-inhibitory peptides. However, Ramchandran and Shah (2008a) have reported that when grown individually, the ACE-inhibitory potential of *S. thermophilus* 1275 and *L. delbrueckii* ssp. *bulgaricus* 1368 was reduced in the presence of 1% Versagel but was improved in the presence of 2% Versagel. Also, with continued proteolysis during storage, some of the active ACE-inhibitory peptides may be hydrolysed and at the same time some new ones may be generated due to the continued proteolytic activity of the starter cultures during storage of the yogurts (Table 4.5). Thus, there is a dynamic equilibrium amongst the

peptides that show ACE-inhibitory potential. A similar observation has been made by Donkor et al. (2007b). There was a high degree of correlation between proteolysis and ACE-inhibition (%) for YV0 (r = 0.94) and YV1 (r = 0.85) but not for YV2. Fuglsang et al. (2003) found a strong correlation between OPA index and *in vitro* ACE-inhibition only for strains of *L. helveticus*.

4.3.5 α-Glu inhibitory activity

Inhibition of α-glu activity is one of the approaches to control post-prandial hyperglycemia. The results of the α-glu inhibitory capability of the TCA filtrates of the selected organisms grown in RSM with and without Versagel and their IC_{50} values are shown in Table 4.7. *Lactobacillus delbrueckii* ssp. *bulgaricus* showed maximum inhibition of α-glu compared to the other organisms grown in RSM. A significant ($P < 0.05$) increase in the inhibitory activity was observed for all organisms grown in RSMV1, except *B. longum* which exhibited a significant ($P < 0.05$) decrease in percent inhibition. Further increases were observed in all organisms grown in RSMV2, except *L. acidophilus* which showed a decrease. These changes were significant ($P < 0.05$) for *L. acidophilus*, *S. thermophilus* and *B. longum*. This implies that LAB could possibly show antidiabetic properties which have not been investigated so far. Yadav et al. (2007) have reported an antidiabetic effect of an Indian fermented milk (dahi) containing *L. acidophilus* and *L. casei*. However, much of the work related to this activity has been for plant parts and some foods that contain certain compounds that can function as α-glu inhibitors (Fujita et al., 2003; Kim et al., 2005; Djomeni et al., 2006; Zhang et al., 2007). So far no work has been carried out on the α-glu inhibitory abilities of LAB.

4.3.6 Production of organic acids

The concentration of lactic and acetic acids produced by the selected organisms in RSM and with added Versagel is presented in Figures 4.2 and 4.3, respectively. As shown in Figure 4.2, *S. thermophilus* produced highest concentration of lactic acid (0.038 mg/mL) while the probiotics produced lowest amount of lactic acid in RSM (Figure 4.2). These corresponded well with the drop in pH and growth observed for these organisms (Table 4.1). The amount of acetic acid produced was highest for *S. thermophilus* (0.021 mg/mL) while the other organisms produced nearly the same quantity of acetic acid (0.017-0.018 mg/mL) in RSM (Figure 4.3). However, the amount of acetic acid produced by *S. thermophilus* was less than that of lactic acid, while *L. delbrueckii* ssp. *bulgaricus*, *L. acidophilus*, *L. casei* and

B. longum produced relatively more acetic acid than lactic acid. Bifidobacteria are able to produce more acetic acid than lactic acid (molar ratio of 3:2) whereas lactobacilli produce more lactic acid (Gardiner et al., 2002). Addition of Versagel resulted in an increase in the quantity of lactic acid produced by *L. casei*, *S. thermophilus* and *B. longum* but a decrease for *L. delbrueckii* ssp. *bulgaricus* in particular. This effect, however, did not match with the pH and growth changes of these organisms except for *L. delbrueckii* ssp. *bulgaricus*. On the other hand, in the presence of Versagel, the organism produced slightly higher quantities of acetic acid, particularly in RSMV2. However, the total amount of acid (lactic+acetic) produced increased with the increase in the concentration of Versagel. The ratio of lactic acid to acetic acid produced was not affected by the addition of Versagel in case of *L. casei* (1:1.1) and *S. thermophilus* (1:0.52). However, this ratio changed for *L. acidophilus* from 1:1.76 in RSM to 1:1.04 and 1:1.22 in RSMV1 and RSMV2 respectively; for *L. delbrueckii* ssp. *bulgaricus* from 1:1.70 in RSM to 1:1.57 in RSMV1 and 1:2.04 in RSMV2 while for *B. longum* it changed from 1:1.14 in RSM to 1:0.66 in both RSMV1 and RSMV2. All these changes indicate a possible shift in the metabolic pathways of these organisms when grown in the presence of Versagel. Lactic acid and acetic acid influence the flavour of fermented milks such as yogurt (Chandan and O'Rell, 2006). Therefore, the ability of the organisms to produce more acetic acid can influence the flavour profile, by enhancing the vinegary note, when used in products such as yogurt.

The concentration of lactic and acetic acids in fresh as well as stored low-fat yogurts is presented in Table 4.8. The concentration of lactic acid was similar in all 3 types of fresh yogurt (4.75-4.87 mg/100 g). While the concentration of lactic acid in YV2 continued to increase significantly ($P < 0.05$) throughout the storage period, significant ($P < 0.05$) increases in YV1 were observed only during the 1st week and at the end of storage at 4 °C. On the other hand, apart from a significant ($P < 0.05$) increase during the 1st week of storage, there was no significant change in the amount of lactic acid in the control yogurts (YV0). However, Donkor et al. (2006) have reported a significant increase in the concentration of lactic acid only in the last week of storage of yogurts prepared from RSM while Amatayakul et al. (2006a) found a slight increase in the concentration of lactic acid throughout the storage period.

The amount of acetic acid produced by the lactic cultures in fresh yogurts was very low, being significantly ($P < 0.05$) lower for YV2 (0.08 mg/100 g) compared to YV1 and YV0 (0.18 mg/100g). There was no significant change in the concentration of acetic acid in any of the three types of yogurt during storage, except at the end of storage (day 28) when a

significant ($P < 0.05$) decrease was observed. Similar to our findings, Donkor et al. (2006) did not observe any change in concentration of acetic acid during storage of yogurts at 4 °C. The amount of total acid (lactic + acetic) was highest in YV2 throughout the storage period, being 6.03 mg/100 g at day 28 followed by 5.69 and 5.35 mg/100 g in YV1 and YV0, respectively. This matches the significant drops in pH observed for these yogurts throughout the storage period (Table 4.4). The high amount of total acid might have resulted in the decrease in counts of *L. delbrueckii* ssp. *bulgaricus* observed towards the end of storage (Table 4.2). The ratio of lactic to acetic acid was 1:0.03 in fresh samples of YV0 and YV1, while it was 1:0.016 in YV2 (Table 4.2) which was due to the slightly higher amount of lactic acid produced in YV2 as compared to YV1 and YV0. This ratio decreased to 1:0.013 in all the samples at the end of storage. Lactic acid bacteria generally produce lactic and acetic acid in the ratio of 3:1 during manufacture of fermented milks such as yogurt, which is considered desirable from the flavour point of view.

4.3.7 Changes in spontaneous whey separation

The spontaneous whey separation (%) of all 3 types of fresh and stored low-fat yogurt is given in Figure 4.4. Addition of Versagel significantly ($P < 0.05$) decreased the spontaneous whey separation in fresh yogurt, being more at 2% level. During storage all samples showed an increase in the amount of whey separation up to 2 weeks of storage at 4 °C. Thereafter, at the end of the storage period, while YV0 and YV1 showed a decrease ($P < 0.05$) in the whey separation, YV2 showed an increase ($P < 0.05$). Al-Kadamany et al. (2003) have also reported a decrease in extent of syneresis in concentrated yogurts towards the end of their storage. Spontaneous whey separation has been related to instability of gel network that results in loss of the ability to entrap all the serum phase (Lucey, 2002). It has been suggested that faster rate of acidification inhibits network rearrangement during whey expulsion thereby resulting in lesser whey separation (Castillo et al., 2006). Considering that yogurts containing Versagel reached the pH of 4.5 faster than control yogurts (Table 4.3), it can be concluded that this could be one of the reasons for lower levels of whey separation in YV1 and YV2 as compared to YV0. Yogurts containing added whey proteins are also reported to have less syneresis (Puvanenthiran et al., 2002; Amatayakul et al., 2006a; Isleten and Karagul-Yuceer, 2006).

4.3.8 Changes in firmness of yogurt gels

The firmness of the 3 types of low-fat yogurt gels, measured after manufacturing and during 28-day cold storage, using the penetration force (g) to break the gels, is shown in Table 4.9. The firmness of yogurt gels containing 2% Versagel was the greatest (82.18 ± SEM g) followed by the control and yogurt containing 1% Versagel (62.64 and 49.75 ± SEM g, respectively). All observed differences were significant ($P < 0.05$). Interestingly, the addition of Versagel at 1% level caused a decrease in the firmness, while at 2% this effect was reversed with a dramatic increase in the firmness compared to the control. Protein-based replacers are known to occupy the spaces that would otherwise be taken up by fat. The smoothness that they impart to the product is due the ball-bearing type of action that occurs around these particles (Singer, 1996). Some reports suggest that addition of whey proteins in yogurt base prior to heat treatment reduces the firmness of the yogurts due to formation of smaller protein aggregates (Puvanenthiran et al., 2002). Furthermore, supplementation of microparticulated whey proteins as fat replacers reduces the firmness of the yogurt because these particles poorly interact with other milk proteins and consequently produce a more open, loose and less dense protein network (Sandoval-Castilla et al., 2004). These could be the reasons for the decrease in firmness of gels when Versagel was added at 1% level (Table 4.9). However, at higher concentration, there must have been a surplus of the replacer particles, and Versagel being a whey protein-based replacer, it appears that there was an increase in protein-protein interactions which resulted in an increase in the firmness of the gel. In an earlier study, it was found that skim milk containing 3% Versagel gelled during sterilization (Ramchandran and Shah, 2008a). Yazici and Akgun (2004) have also found an increase in hardness of yogurts with increasing levels of protein-based fat replacers.

During storage, all the 3 types of yogurt showed a significant ($P<0.05$) increase in firmness during the 1st week of storage. While the firmness of the control remained fairly constant during the remaining period of storage, it even further increased significantly ($P < 0.05$) for the 2nd week of storage in YV1 and YV2. Yazici and Akgun (2004) also observed increases in hardness of yogurts containing protein-based fat replacers during storage at 4-6 °C.

4.3.9 Viscoelastic properties of fresh yogurts

The viscoelastic properties of the 3 types of fresh low-fat yogurt were assessed using a small amplitude oscillatory measurement and results are presented in Figure 4.5. The flow behaviour of the samples under increasing shear rate was modelled using the Power Law and

Herschel-Bulkley models. The coefficients associated with these two models are presented in Table 4.10. Addition of Versagel resulted in an increase in G′ values (Figure 4.5) which indicates that Versagel imparted more solid-like (elastic) properties to the low-fat yogurt. The increase was very apparent upon 2% addition of Versagel with the maximum of 191 Pa as opposed to 1% addition and control with 103.8 and 95.2 Pa, respectively (Figure 4.5). Similarly, tan δ values for YV2 sample was the lowest followed by YV0 and YV1, another indicator of more solid like properties. Heating of milk enriched with whey protein ingredients leads to greater cross linking and therefore imparts more solid-like properties to the gel (Remeuf et al., 2003) which could be observed in the yogurts containing Versagel. Patocka et al. (2006) have found that decrease in the G′ values of whey protein supplemented yogurt could be rationalized in terms of a network weakening. Moreover, an increase in the G′ values in yogurts containing Versagel as compared to the control (Figure 4.5) indicated continuity of the network implying that stronger gels were formed (Patocka et al., 2006). The yield stress also increased in yogurts containing Versagel, being higher in YV2 ($P < 0.05$) than those in YV1 and control (Table 4.10). This concurred with the higher firmness observed for YV2 (Table 4.9). Both consistency and yield stress showed a good correlation with the firmness of the yogurts ($r = 0.77$ and 0.99, respectively). The cross-linking capacity of denatured whey proteins plays an important role in the organization of the yogurt structure by enhancing the degree of bridging between protein particles (Remeuf et al., 2003). The proteins in most of the microparticulated fat replacers such as Versagel are denatured during their manufacture (Singer, 1996). This may explain the increase in yield stress observed in the presence of Versagel. The yield stress of YV1 was lower than that of the control (YV0) which further supports the lower firmness observed in YV1 compared to YV0 (Table 4.9). Furthermore, the consistency index as estimated by the Power Law increased in yogurts containing Versagel, being higher ($P < 0.05$) in YV2 than those in YV1 and the control yogurts (Table 4.10). These results indicate that the addition of Versagel improved the consistency of the products in comparison to the control. These products were more elastic with distinct pseudoplastic properties (YV2 > YV1) and greater ability to resist deformation upon applied shear. The Herschel Bulkley model appeared to provide a better fit to the experimental data than the Power law model as shown by the higher R^2 values (Table 4.10).

4.4 Conclusion

Addition of Versagel resulted in improved growth of *S. thermophilus* and *B. longum* but inhibited that of *L. casei*, *L. acidophilus* and *L. delbrueckii* ssp. *bulgaricus*. This is reflected in the extent of reduction of pH by these organisms. Among the biochemical activities, proteolytic activity of almost all the organisms except *B. longum*, was adversely affected by the presence of Versagel although the ACE-inhibitory and α-glu inhibitory activities were improved. The rate of addition of Versagel mainly influenced the growth, ACE-inhibitory and α-glu inhibitory activities of the selected organisms. Thus, it is appears that additives such as Versagel can influence the growth and related biochemical activities of organisms in fermented milks.

Incorporation of Versagel as a fat replacer in the preparation of low-fat yogurt decreased the fermentation time during yogurt making. The pH of yogurts containing Versagel was higher than the control while the concentrations of lactic and acetic acids were similar in the fresh samples of low-fat yogurt regardless of the presence of Versagel. Although there was an increase in the concentration of lactic acid in the 3 types of yogurt, there was no change in the level of acetic acid during the storage of the yogurts. The starter cultures, *S. thermophilus* 1275 and *L. delbrueckii* ssp. *bulgaricus* 1368, maintained their viability in all the yogurts throughout the storage period. However, the proteolytic and ACE-inhibitory potential of the starter cultures was suppressed in the presence of Versagel. On the other hand, the addition of Versagel had a positive impact on the physical properties of the low-fat yogurt. The amount of spontaneous whey separation reduced while firmness and pseudoplastic properties improved in low-fat yogurts containing Versagel. Thus, Versagel can be useful in improving the textural characteristics of low-fat yogurt but has a negative impact on proteolytic and ACE-inhibitory potential.

Table 4.1 Changes in pH, log counts and OPA values (absorbance at 340 nm) during growth of *L. casei*, *L. acidophilus*, *S. thermophilus*, *L. delbrueckii* ssp. *bulgaricus* and *B. longum* in reconstituted skim milk (RSM) with and without Versagel, for 6 h at 37 °C.

	Delta pH			Delta $\log_{10}$ CFU/mL			ΔA_{340}		
	RSM	RSMV1	RSMV2	RSM	RSMV1	RSMV2	RSM	RSMV1	RSMV2
LC	0.11 ± 0.02^{a}	0.15 ± 0.01^{a}	0.13 ± 0.01^{a}	0.86 ± 0.04^{a}	0.94 ± 0.06^{a}	0.82 ± 0.16^{a}	0.024 ± 0.002^{a}	0.025 ± 0.002^{a}	0.010 ± 0.002^{b}
LA	0.11 ± 0.00^{a}	0.11 ± 0.00^{a}	0.10 ± 0.00^{b}	0.46 ± 0.13^{a}	0.26 ± 0.03^{b}	0.26 ± 0.03^{b}	0.006 ± 0.001^{a}	0.004 ± 0.001^{b}	0.005 ± 0.001^{b}
S.T	1.06 ± 0.00^{a}	1.11 ± 0.00^{b}	1.09 ± 0.00^{c}	1.96 ± 0.10^{a}	2.11 ± 0.05^{b}	2.13 ± 0.03^{b}	0.048 ± 0.002^{a}	0.015 ± 0.010^{b}	0.004 ± 0.001^{c}
LB	0.36 ± 0.02^{a}	0.24 ± 0.00^{b}	0.26 ± 0.00^{b}	1.22 ± 0.07^{a}	1.07 ± 0.06^{b}	1.10 ± 0.08^{b}	0.047 ± 0.002^{a}	0.000 ± 0.000^{b}	0.000 ± 0.000^{b}
BL	0.13 ± 0.01^{a}	0.12 ± 0.00^{a}	0.12 ± 0.00^{a}	0.33 ± 0.11^{a}	0.39 ± 0.15^{a}	0.47 ± 0.13^{a}	0.003 ± 0.001^{a}	0.022 ± 0.004^{b}	0.046 ± 0.003^{c}

Results presented are means ± SD of 6 observations at 95% level of confidence.
RSMV1 is RSM containing 1% Versagel; RSMV2 is RSM containing 2% Versagel
[ab]Means in the same row of each parameter with different superscripts are significantly different.
LC = *L. casei* 15286, LA = *L. acidophilus* 4461, ST = *S. thermophilus* 1275, LB = *L. delbrueckii* ssp. *bulgaricus* 1368, BL = *B. longum* 5022

Table 4.2 Counts (log CFU/g) of *S. thermophilus* 1275 and *L. delbrueckii* ssp. *bulgaricus* 1368 in low-fat yogurts with or without Versagel stored at 4 °C for 28 d.

	Storage period			
	Day 1	Day 7	Day 14	Day 28
S. thermophilus 1275				
YV0	8.68^{aA}	8.72^{aA}	8.71^{aA}	8.73^{aA}
YV1	8.64^{aA}	8.77^{bA}	8.81^{bB}	8.79^{bA}
YV2	8.70^{aA}	8.72^{aA}	8.79^{bB}	8.72^{aA}
SEM	0.03			
L. delbrueckii ssp. *bulgaricus* 1368				
YV0	8.74^{aA}	8.73^{aA}	8.70^{aA}	8.60^{bA}
YV1	8.73^{aA}	8.81^{aA}	8.79^{aB}	8.51^{bB}
YV2	8.73^{aA}	8.74^{aA}	8.81^{aB}	8.58^{bBA}
SEM	0.03			

Values are the means of six observations at 95% level of confidence
SEM = standard error of means
YV0 = control yogurt prepared from skim milk standardized to 12% total solids; YV1 = yogurt prepared from skim milk standardized to 12% total solids and added with 1% (w/v) Versagel; YV2 = yogurt prepared from skim milk standardized to 12% total solids and added with 2% (w/v) Versagel
[ab]Means in the same row with different alphabets are significantly different within a particular treatment for each organism
[AB]Means in the same column with different alphabets are significantly different for a particular day of storage for each organism

Table 4.3 Average of changes in pH of skim milk with and without Versagel during manufacture of low fat yogurt at 42 °C.

Period of incubation	Type of yogurt		
	YV0	YV1	YV2
0 h	6.51	6.44	6.38
3 h	5.89	5.84	5.81
3 h 30 min	5.49	5.48	5.27
4 h	5.09	4.93	4.83
4 h 30 min	4.77	4.64	4.61
4 h 40 min	4.60	4.51	4.48
5 h	4.55	-	-

Values presented are average of three replicates
YV0 = control yogurt prepared from skim milk standardized to 12% total solids; YV1 = yogurt prepared from skim milk standardized to 12% total solids and added with 1% (w/v) Versagel; YV2 = yogurt prepared from skim milk standardized to 12% total solids and added with 2% (w/v) Versagel

Table 4.4 pH of low-fat yogurts with or without Versagel and changes during storage 4 °C for 28 d.

	Storage period			
Type of Yogurt	Day 1	Day 7	Day 14	Day 28
YV0	4.47^{aA}	4.31^{bA}	4.29^{bA}	4.29^{bA}
YV1	4.55^{aB}	4.34^{bA}	4.30^{bA}	4.25^{bcA}
YV2	4.56^{aB}	4.36^{bB}	4.26^{cA}	4.26^{cA}
SEM	0.018			

Values are the means of six observations at 95% level of confidence
SEM = standard error of means
YV0 = control yogurt prepared from skim milk standardized to 12% total solids; YV1 = yogurt prepared from skim milk standardized to 12% total solids and added with 1% (w/v) Versagel; YV2 = yogurt prepared from skim milk standardized to 12% total solids and added with 2% (w/v) Versagel
[abc]Means in the same row with different alphabets are significantly different within a particular treatment
[AB]Means in the same column with different alphabets are significantly different for a particular day of storage

Table 4.5 Proteolysis (Δ absorbance at 340 nm) in low-fat yogurts with or without Versagel stored at 4 °C for 28 d.

	Storage period			
Type of Yogurt	Day 1	Day 7	Day 14	Day 28
YV0	0.386^{aA}	0.437^{aA}	0.527^{bA}	0.945^{bcA}
YV1	0.336^{aA}	0.444^{bA}	0.486^{bA}	0.540^{bcB}
YV2	0.487^{aB}	0.595^{bB}	0.580^{bAB}	0.756^{cC}
SEM	0.024			

Values are the means of six observations at 95% level of confidence
SEM = standard error of means
YV0 = control yogurt prepared from skim milk standardized to 12% total solids; YV1 = yogurt prepared from skim milk standardized to 12% total solids and added with 1% (w/v) Versagel; YV2 = yogurt prepared from skim milk standardized to 12% total solids and added with 2% (w/v) Versagel
^{abc}Means in the same row with different alphabets are significantly different within a particular treatment
^{ABC}Means in the same column with different alphabets are significantly different for a particular day of storage

Table 4.6 ACE-inhibition (%) and IC_{50} (mg/mL) of TCA filtrates of *L. casei*, *L. acidophilus*, *S. thermophilus*, *L. delbrueckii* ssp. *bulgaricus* and *B. longum* grown in reconstituted skim milk (RSM) with and without Versagel for 6 h at 37 ºC.

Organisms	RSM		RSMV1		RSMV2	
	ACEI	IC_{50}	ACEI	IC_{50}	ACEI	IC_{50}
L. casei 15286	25.21 ± 2.40[a]	0.38	34.78 ± 2.61[b]	0.54	41.17 ± 3.70[c]	0.50
L. acidophilus 4461	39.32 ± 2.44[a]	0.34	39.52 ± 3.51[a]	0.44	43.99 ± 1.94[b]	0.42
S. thermophilus 1275	38.20 ± 0.66[a]	0.27	23.34 ± 1.81[b]	0.66	41.09 ± 2.43[c]	0.40
L. delbrueckii ssp. *bulgaricus* 1368	40.39 ± 4.01[a]	0.36	31.97 ± 1.47[b]	0.57	42.58 ± 3.73[a]	0.47
B. longum 5022	41.39 ± 3.53[a]	0.30	27.81 ± 1.87[b]	0.59	42.41 ± 1.38[a]	0.40

Results presents are means ± SD of 6 observations.
RSMV1 is RSM containing 1% Versagel, RSMV2 is RSM containing 2% Versagel
[ab]Means in the same row of each parameter with different superscripts are significantly different.

Table 4.7 α-Glucosidase inhibition(%) and IC_{50} (mg/mL) of TCA filtrates of *L. casei*, *L. acidophilus*, *S. thermophilus*, *L. delbrueckii* ssp. *bulgaricus* and *B. longum* grown in reconstituted skim milk (RSM) with and without Versagel for 6 h at 37 ºC.

Organisms	RSM		RSMV1		RSMV2	
	α-Glu inhibition	IC_{50}	α-Glu inhibition	IC_{50}	α-Glu inhibition	IC_{50}
L. casei 15286	62.29 ± 1.45^{a}	0.15	77.28 ± 2.30^{b}	0.24	76.87 ± 3.02^{b}	0.27
L. acidophilus 4461	63.83 ± 1.49^{a}	0.21	76.54 ± 2.39^{b}	0.23	68.09 ± 1.66^{c}	0.27
S. thermophilus 1275	65.84 ± 2.08^{a}	0.16	73.83 ± 4.03^{b}	0.21	92.77 ± 2.73^{c}	0.18
L. delbrueckii ssp. *bulgaricus* 1368	73.45 ± 3.45^{a}	0.20	70.89 ± 5.82^{ab}	0.26	64.25 ± 3.09^{b}	0.31
B. longum 5022	67.55 ± 2.54^{a}	0.18	66.15 ± 2.09^{b}	0.25	68.61 ± 2.35^{a}	0.25

Results presents are means ± SD of 6 observations.
RSMV1 is RSM containing 1% Versagel, RSMV2 is RSM containing 2% Versagel
[ab]Means in the same row of each parameter with different superscripts are significantly different.

Table 4.8 Concentration of lactic and acetic acids (mg/100g) in low-fat yogurts with or without Versagel stored at 4 °C for 28 d.

	Storage period			
	Day 1	Day 7	Day 14	Day 28
Lactic acid				
YV0	4.77^{aA}	5.08^{bA}	5.07^{bA}	5.28^{bA}
YV1	4.75^{aA}	5.16^{bA}	5.35^{bB}	5.62^{cB}
YV2	4.87^{aA}	5.28^{bA}	5.63^{cC}	5.95^{dC}
SEM	0.08			
Acetic acid				
YV0	0.18^{aA}	0.17^{aA}	0.10^{abA}	0.07^{bA}
YV1	0.18^{aA}	0.13^{aA}	0.12^{aA}	0.07^{bA}
YV2	0.08^{aB}	0.09^{aA}	0.11^{aA}	0.08^{aA}
SEM	0.03			

Values are the means of six observations less the 0 h concentrations at 95% level of confidence

SEM = standard error of means

YV0 = control yogurt prepared from skim milk standardized to 12% total solids; YV1 = yogurt prepared from skim milk standardized to 12% total solids and added with 1% (w/v) Versagel; YV2 = yogurt prepared from skim milk standardized to 12% total solids and added with 2% (w/v) Versagel

^{abc}Means in the same row with different alphabets are significantly different within a particular treatment for each organic acid

^{ABC}Means in the same column with different alphabets are significantly different for a particular day of storage for each organic acid

Table 4.9 Firmness (g) of low-fat yogurts prepared with or without Versagel during 28 d cold (4 °C) storage.

	Storage period			
Type of Yogurt	Day 1	Day 7	Day 14	Day 28
YV0	62.64^{aA}	71.89^{bA}	72.39^{bA}	73.52^{bA}
YV1	49.75^{aB}	55.57^{bB}	60.43^{bcB}	60.98^{bcB}
YV2	82.18^{aC}	91.95^{bC}	98.52^{cC}	101.26^{cC}
SEM	1.71			

Values are the means of six observations at 95% level of confidence
SEM = standard error of means
YV0 = control yogurt prepared from skim milk standardized to 12% total solids; YV1 = yogurt prepared from skim milk standardized to 12% total solids and added with 1% (w/v) Versagel; YV2 = yogurt prepared from skim milk standardized to 12% total solids and added with 2% (w/v) Versagel
^{abc}Means in the same row with different alphabets are significantly different within a particular treatment;
^{ABC}Means in the same column with different alphabets are significantly different for a particular day of storage

Table 4.10 Flow behaviour of fresh low-fat yogurts prepared by addition of 1 or 2% Versagel predicted by the Ostwald (Power law) and Herschel-Bulkley models.

	Power Law model			Herschel-Bulkley model			
	k (Pa.s)	n	R^2	σ_0 (Pa)	k (Pa.s)	n	R^2
YV0	6.16[a]	0.414[a]	0.985	5.47[a]	2.56[a]	0.601[a]	0.987
YV1	7.67[a]	0.386[b]	0.992	3.22[a]	6.07[a]	0.439[a]	0.994
YV2	11.39[ba]	0.365[c]	0.977	12.26[ab]	2.68[a]	0.669[a]	0.990
SEM	0.95	0.005		1.91	0.93	0.063	

Values are the means of six observations at 95% level of confidence
SEM = standard error of means
YV0 = control yogurt prepared from skim milk standardized to 12% total solids; YV1 = yogurt prepared from skim milk standardized to 12% total solids and added with 1% (w/v) Versagel; YV2 = yogurt prepared from skim milk standardized to 12% total solids and added with 2% (w/v) Versagel
[ab]Means in the same column with different alphabets are significantly different within a particular parameter

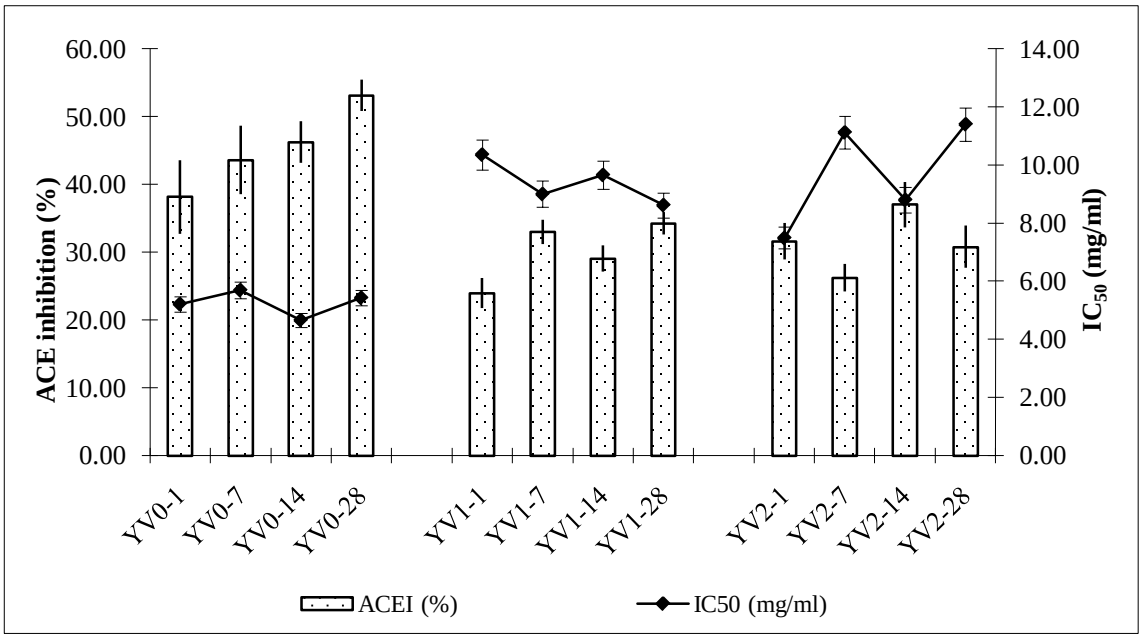

Figure 4.1 Changes in ACE-inhibition (%) and corresponding IC_{50} (mg/ml) values of fresh and stored (4 °C for 28 d) low-fat yogurts with or without Versagel. YV0 = control yogurt prepared from skim milk standardized to 12% total solids; YV1 = yogurt prepared from skim milk standardized to 12% total solids and added with 1% (w/v) Versagel; YV2 = yogurt prepared from skim milk standardized to 12% total solids and added with 2% (w/v) Versagel. Subscript 1 to 28 following the yogurt types indicates storage period D1 to D28 (Error bars represent the pooled standard error of the means)

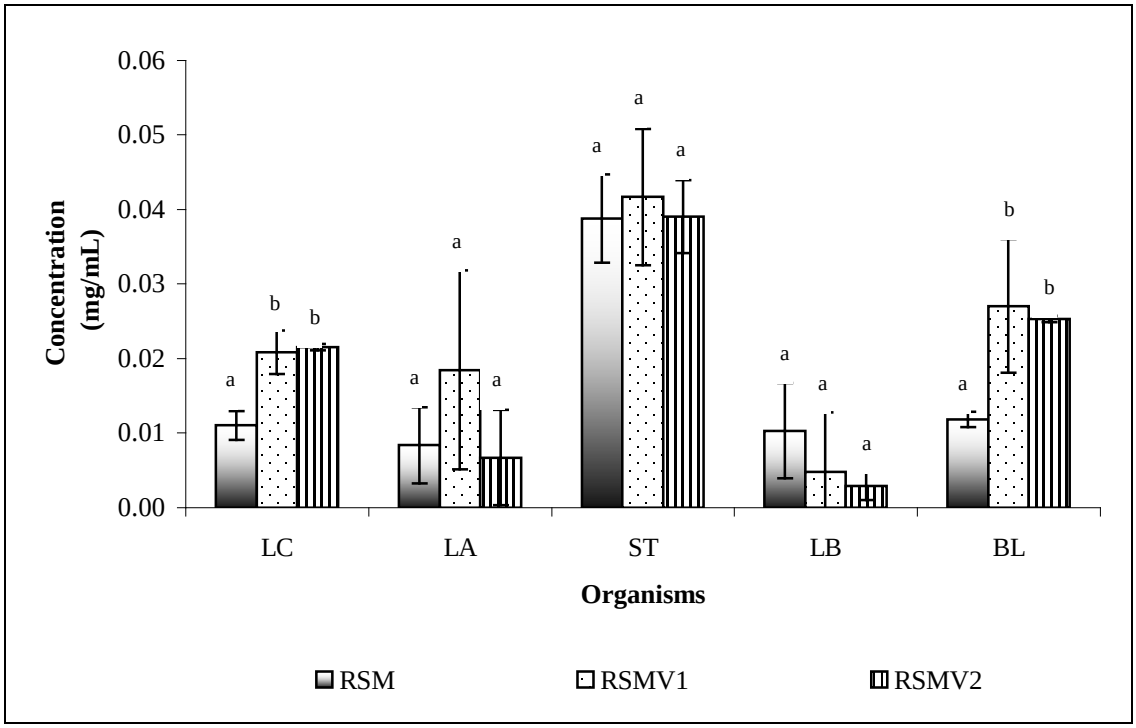

Figure 4.2 Concentration of lactic acid (mg/mL) produced by *L. casei*, *L. acidophilus*, *S. thermophilus*, *L. delbrueckii* ssp. *bulgaricus* and *B. longum* grown at 37 ºC for 6 h in reconstituted skim milk (RSM) with and without Versagel. LC = *L. casei* 15286, LA = *L. acidophilus* 4461, ST = *S. thermophilus* 1275, LB = *L. delbrueckii* ssp. *bulgaricus* 1368, BL = *B. longum* 5022. RSMV1 is RSM containing 1% Versagel, RSMV2 is RSM containing 2% Versagel (Error bars represent the pooled standard error of the means. [ab]Means of bars of the same organism with different superscripts are significantly different)

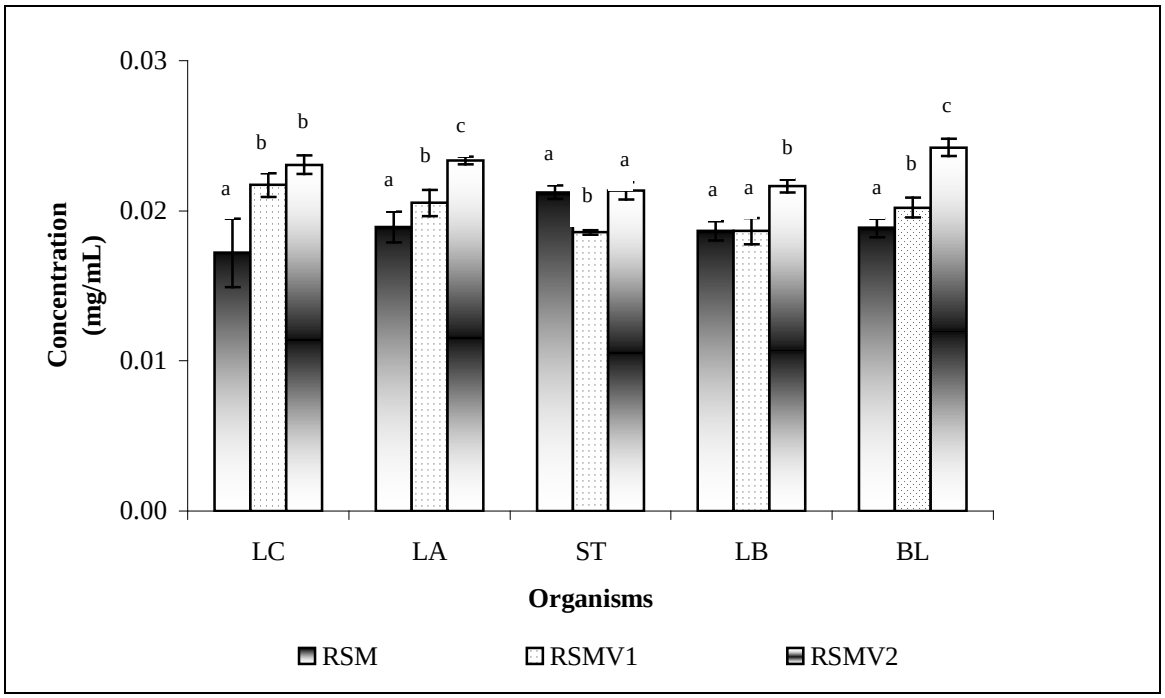

Figure 4.3 Concentration of acetic acid (mg/mL) produced by *L. casei*, *L. acidophilus*, *S. thermophilus*, *L. delbrueckii* ssp. *bulgaricus* and *B. longum* grown at 37 ºC for 6 h in reconstituted skim milk (RSM) with and without Versagel. LC = *L. casei* 15286, LA = *L. acidophilus* 4461, ST = *S. thermophilus* 1275, LB = *L. delbrueckii* ssp. *bulgaricus* 1368, BL = *B. longum* 5022. RSMV1 is RSM containing 1% Versagel, RSMV2 is RSM containing 2% Versagel (Error bars represent the pooled standard error of the means. ^{abc}Means of bars of the same organism with different superscripts are significantly different)

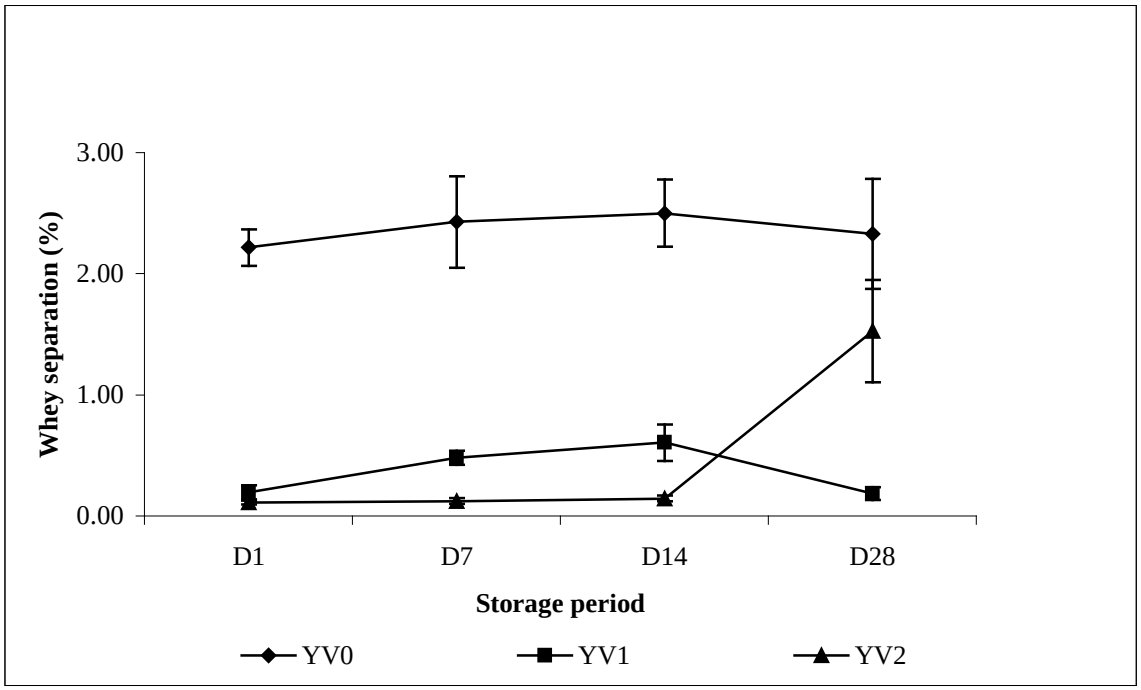

Figure 4.4 Changes in spontaneous whey separation (%) in fresh and stored (4 °C for 28 d) low-fat yogurts with or without Versagel. YV0 = control yogurt prepared from skim milk standardized to 12% total solids; YV1 = yogurt prepared from skim milk standardized to 12% total solids and added with 1% (w/v) Versagel; YV2 = yogurt prepared from skim milk standardized to 12% total solids and added with 2% (w/v) Versagel (Error bars represent the pooled standard error of the means)

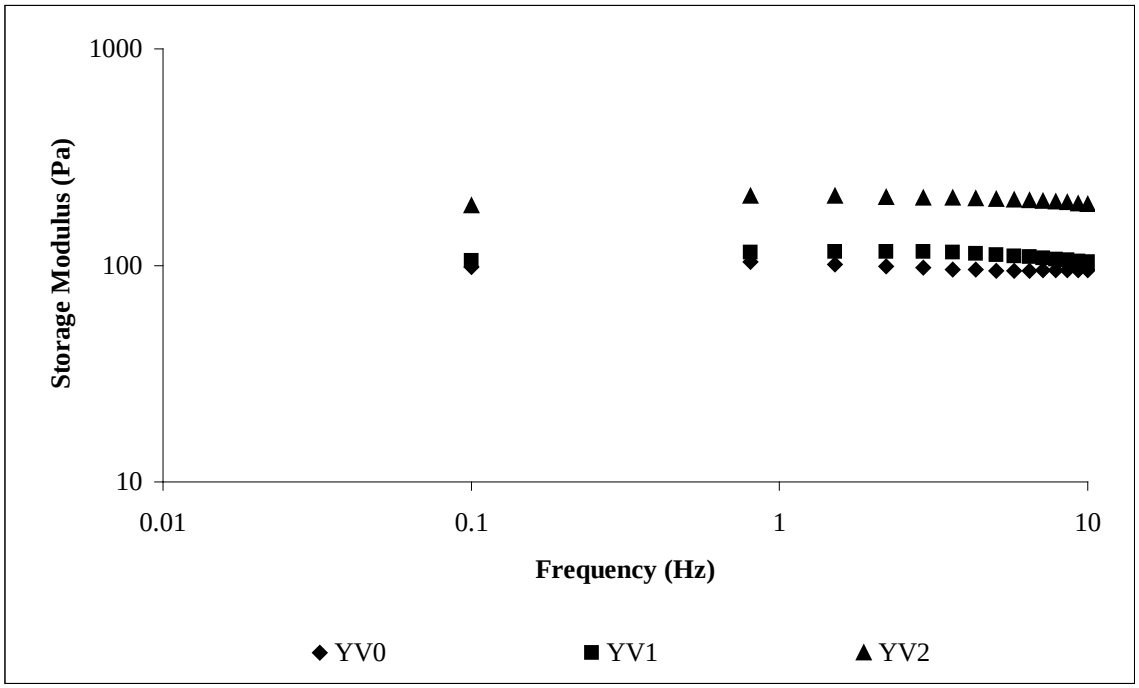

Figure 4.5 Elastic modulus (G′) of low-fat yogurts prepared with or without Versagel after manufacturing. YV0 = control yogurt prepared from skim milk standardized to 12% total solids; YV1 = yogurt prepared from skim milk standardized to 12% total solids and added with 1% (w/v) Versagel; YV2 = yogurt prepared from skim milk standardized to 12% total solids and added with 2% (w/v) Versagel.

5.0 Influence of Addition of Raftiline HP® on the Growth, Proteolytic, ACE- and α-Glucosidase Inhibitory Activities of Selected Lactic Acid Bacteria and *Bifidobacterium* and on Microbial, Chemical and Physical Properties of Low-fat Yogurt

A version of this chapter has been published.

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5.1 Introduction

Lactic acid bacteria (LAB) are Gram-positive, non-sporing, catalase-negative organisms that are aero-tolerant, fastidious, acid-tolerant, strictly fermentative, and produce lactic acid as the major end product of sugar fermentation (Axelsson, 1998). Several strains of LAB have gained importance in recent years, in regard to food and nutrition, which include the starter organisms as well as the probiotics. Yogurt with probiotic organisms is a classic example of a functional food (Vierhile, 2006). Apart from the health benefits accruing from the direct consumption of bacteria from yogurt, these organisms also produce a range of secondary metabolites which have been associated with health-promoting properties. These include the bioactive peptides which are peptides with hormone- or drug-like activity that eventually modulate specific physiological functions of the human body. The bioactivities of peptides released from milk proteins include hypotensive, immuno-modulatory, anticancer, hypocholesterolaemic, antimicrobial, opioid and mineral-binding (Gobbetti et al., 2002; Fitzgerald and Murray, 2006; Korhonen and Pihlanto, 2006). The most probable liberation of bioactive peptides occurs via proteolysis and microbial enzymes. The potential of LAB to generate such peptides has been studied extensively in recent years (Gobbetti et al., 2000; Korhonen and Pihlanto, 2006).

Fermented functional foods are a popular category of foods that promises the combined benefits of health and nutrition. These foods contain components that are categorized as probiotics and prebiotics. Several manufacturers have capitalised on the health promoting properties of products containing probiotics and prebiotics (Tomasik and Tomasik, 2003; Fitzgerald and Murray, 2006). This, however, requires careful selection of starter cultures and/or probiotics to ensure *in situ* expression of desirable health beneficial metabolites in addition to maintaining the product quality (Leroy and De Vuyst, 2004).

The level of fat determines the nutritional, physical, chemical and sensory characteristics of foods. The universal awareness of the adverse effects of excessive dietary fat intake has modified the diets of health conscious consumers leading to a flood of low-fat or no-fat foods in the market. The threat of obesity and related diseases has popularised low-fat and sugar-free products in the market. This, however, necessitates the use of ingredients to maintain the product quality in such foods. The terms that are used to describe such ingredients that can replace fat are fat replacer, fat substitute, fat mimetic, low-calorie fat and fat extender (Jones, 1996a). Fat mimetics are substances that imitate organoleptic or physical properties of triglycerides but which cannot replace fat on a one-to-one, gram-for-gram basis

(Akoh, 1998). Recently, the food industry as well as the consumers have shown a growing interest in functional foods. These are foods that provide health benefits beyond basic nutrition (Spence, 2006). On a broader spectrum, all foods with reduced fat content can be considered as functional foods given the nutritional and health benefits of fat reduction.

Replacement of native milk constituents, particularly milk fat, in the low-fat functional fermented milks often results in a modified texture or mouth-feel that can detract its consumption. As a result, a number of ingredients have been commercialized that can overcome these defects. Among the various ingredients used to replace fat in low-fat or no-fat foods, fiber-based mimetics provide special positive physiological benefits since there is a growing recognition for the role of dietary fiber in disease prevention (Jones, 1996a). Most popular in this category of food additives are inulin and oligofructose. Inulin, a natural food ingredient found in many vegetables, is obtained industrially from chicory roots. Inulin type fructans are composed of β-D-fructofuranoses attached by β→1|2 linkages. As a consequence of the potential health promoting properties of this ingredient, it has been included under the head of prebiotics and is considered to be a functional food ingredient. A prebiotic is “a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon that can improve the host health” (Roberfroid et al., 1998).

Inulin and oligofructose commercially available today are either synthesized from sucrose or extracted from chicory roots which contain ~ 15-20% inulin and 5-10% oligofructose (Niness, 1999). Generally the product with a degree of polymerization (DP) from 2 to 60+ is labelled inulin. The inulin from which small molecular weight oligomers have been eliminated is called inulin high performance (HP) (Roberfroid, 1999a, Murphy, 2001). HP inulin has a DP ranging from 11 to 60, averaging 25. This product provides almost twice the fat mimetic characteristics of standard inulin without contributing to sweetness. It is a common ingredient of choice in low-fat products where a creamy, fat-like mouthfeel with no added sweetness is desired. Several fat mimetics, such as Raftiline, have been launched based on fiber from a number of different sources of which inulin based fat replacers have shown to have positive physiological benefits arising from their bifidogenic properties (Jones, 1996a; Jenkins et al., 1999). In addition to being highly heat stable, inulin has high solubility and stability in acidic conditions; this makes this ingredient suitable for fortifying dairy products, particularly fermented dairy foods such as yogurt. Apart from these technological benefits, use of inulin also provides some nutritional and health benefits that

include its bifidogenic effect and its function as a prebiotic. Hence, some yogurt manufacturers have included inulin in their product (Niness, 1999).

Yogurt is traditionally considered to be a healthy food. It can be made functional by the addition of probiotics and prebiotics. The functionality of yogurt is further enhanced by the release of bioactive peptides during lactic fermentation (Shah, 2007). These peptides are encrypted in the milk proteins and are released during fermentation due to the proteolytic activities of the organisms used. Among the various peptides released, the ones that show an antihypertensive activity due to their ability to suppress the activity of angiotensin-I converting enzyme (ACE) have held the interest of researchers and consumers (Gobbetti et al., 2002; Fitzgerald et al., 2004; Donkor et al., 2007b). There has been considerable interest in preparing yogurt with inulin as added prebiotic. Generally inulin is used at 2 to 5% of the formulation for both nutritional and functional benefits in yogurt. Most of the research work conducted so far has been related to the textural, rheological and sensory attributes of low-fat or no fat yogurt containing inulin (Robinson, 1995; Dello Staffolo et al., 2004; Guven et al., 2005; Seydm et al., 2005; Kip et al., 2006; Tarrega and Costell, 2006; Aryana et al., 2007).

Most of the studies related to inclusion of inulin in yogurt have examined its influence on the sensory properties and texture of the product (Guven et al., 2005; Kip et al., 2006; Tarrega and Costell, 2006; Aryana et al., 2007) and very little has been reported on the growth and metabolism of the microorganisms included in the product (Özer et al., 2005; Ramchandran and Shah, 2008b). Some pure culture studies have been conducted on its effect on bifidobacteria (Roberfroid et al., 1998; Rossi et al., 2005).

Although manufacturers now use inulin commonly as a fat replacer in low-fat fermented milks such as yogurt, there is a gap in literature with regards to studies on the influence of inulin on some important biochemical activities such as proteolysis, organic acid production and growth of organisms used in yogurt making as well as ACE-inhibitory activity. As yet no work has been carried out on the biochemical aspects of yogurt cultures as well as on the textural benefits due to addition of inulin in yogurt. Therefore, this study was designed with the aims of i) understanding the effect of adding varying levels of a commercially available HP inulin, Raftiline HP® (now known as Beneo HP®), on the growth, proteolytic activity, angiotensin-I converting enzyme (ACE) and α-glucosidase (α-glu) inhibitory activities, and production of organic acids by selected strains of *S. thermophilus*, *L. delbrueckii* ssp. *bulgaricus* and probiotic organisms when grown individually and ii) examining the influence of added Raftiline HP on the pH of yogurt mix during manufacturing, growth of starter bacteria *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus*,

proteolytic and ACE-inhibitory activities, organic acid production, firmness, spontaneous whey separation and rheological properties of low fat yogurt.

5.2 Materials and Methods

5.2.1 Microorganisms and their activation

The organisms used have been described under Section 4.2.1 and their method of activation has been described under Section 3.2.1.

5.2.2 Preparation of Raftiline HP added RSM

Raftiline HP was added at the rate of 1, 2 and 3% (w/v) to RSM (12 %, w/v) and the RSM was then autoclaved at 121 °C for 15 min. Raftiline HP was supplied by Orafti Ingredients, Malvern, PA, USA.

5.2.3 Preparation of low-fat yogurt

Three batches of low-fat yogurts were prepared from skimmed milk (Skinny Milk, Parmalat Foods Pty Ltd., Vic) standardized to 12% total solids using skim milk powder added at 60 °C. The control (YI0) batch was prepared from the standardized skim milk. The experimental batches of yogurts (YI2 and YI3) were prepared from standardized skim milk added with 2% and 3% Raftiline HP (w/v), respectively. Raftiline HP was supplied by Orafti Ingredients, Malvern, PA, USA. The 2 levels of Raftiline HP (2 and 3%) were selected based on proteolytic and ACE-inhibitory activities of the cultures when grown individually in the presence of varying levels of Raftiline HP. The cultures showed maximum-ACE inhibitory activity in the presence of 2 and 3% Raftiline HP (Ramchandran and Shah, 2009b). Raftiline HP was added when the milk temperature reached 80 °C, since the solubility of inulin improves with temperature, being less soluble at room temperature (25°C) (Kim and Wang, 1999). The method of yogurt making and sampling is described under Section 4.2.3.

5.2.4 Estimation of growth

The growth was observed for all organisms in RSM as well as in RSM containing 1 (RSMR1), 2 (RSMR2) and 3 (RSMR3) % Raftiline HP at 37 °C as described in Section 3.2.2. The results were expressed as $\log_{10}$ colony forming units (CFU) per millilitre of sample and increase in counts was calculated by subtracting $\log_{10}$ counts at 0 h from those at 6 h.

The growth and viability of the yogurt starters were performed as described in Section 4.2.4.

5.2.5 Measurement of pH

The pH of all RSM samples was measured as described in Section 3.2.2. Drop in pH was the difference between the two observations at 0 h and 6 h.

The drop in pH during preparation of the 3 types of low-fat yogurt and during 28 days storage at 4 °C was measured as indicated under Section 4.2.5.

5.2.6 Preparation of filtrates for biochemical analyses

The trichloroacetic acid (TCA) filtrates of RSM samples, removed at 0 and 6 h of incubation, was prepared and stored as described in Section 3.2.3.

Filtrates of low-fat yogurt samples stored at 4 °C were prepared and stored as described in Section 4.2.6.

5.2.7 Determination of extent of proteolysis

Proteolytic activity of all cultures grown in RSM was determined as described in Section 3.2.4. The increase in free amino acids content was calculated by subtracting the absorbance at 0 h from that at 6 h.

The extent of proteolysis in low-fat yogurts was also determined as described in Section 3.2.4 and calculated as in Section 4.2.7.

5.2.8 Measurement of ACE-inhibitory activity

The ACE-inhibitory activity of the TCA filtrates of cultures after 6 h at 37 °C as well as the protein content was determined as described in Section 3.2.6.

The ACE-inhibitory activity was determined in the filtrates of freshly inoculated mixes (0 h) as well as of the 3 types of low-fat yogurt as described in Section 3.2.6. The protein content was calculated as indicated in Section 4.2.8.

5.2.9 Estimation of alpha-glucosidase inhibitory activity

The α-glucosidase (α-glu) inhibitory activity of the TCA filtrates of organisms after 6 h of growth at 37 °C was measured as described in Section 3.2.7.

5.2.10 Determination of organic acids

The acetic and lactic acids contents in the RSM samples incubated at 37 °C for 0 and 6 h as well as those in all the 3 types of low-fat yogurt samples stored at 4 °C as well as the 0 h samples of mixes were determined by the method described in Section 4.2.10.

5.2.11 Spontaneous whey separation

Spontaneous whey separation in the stored low-fat yogurt samples was measured by the siphon method described in Section 6.2.9.

5.2.12 Firmness of yogurt gels

The firmness of the low-fat yogurts was determined using a texture analyzer as described in Section 6.2.10.

5.2.13 Rheological measurements

The viscoelastic properties of the fresh low-fat yogurt samples were determined by the procedure described in Section 6.2.11.

5.2.14 Statistical analysis

Statistical analyses of data obtained from RSM fermentations were performed as described in Section 3.2.8. Statistical analyses of data obtained for low-fat yogurt were performed as described in Section 4.2.14.

5.3 Results and Discussion

5.3.1 Growth and pH

Changes in $\log_{10}$ count and pH observed during the 6 h growth of the organisms in RSM with and without Raftiline HP are presented in Table 5.1. Although all the organisms, except *L. acidophilus* and *L. delbrueckii* ssp. *bulgaricus*, showed a better growth in the presence of 1% Raftiline HP than that in RSM, no corresponding effect on the decline in pH of the medium was observed. On the contrary, no significant ($P > 0.05$) decrease in delta pH values was exhibited by *L. casei* and *S. thermophilus* grown in RSMR1 compared to RSM while *L. delbrueckii* ssp. *bulgaricus* and *B. longum* showed a significantly ($P < 0.05$) less drop in pH. However, none of the organisms showed any change in pH in RSMR2 in comparison to RSMR1 except *L. casei* which showed a significant ($P < 0.05$) drop in pH.

There was no further significant ($P > 0.05$) decrease in pH for any of the organisms when the level of Raftiline HP was increased to 3% (RSMR3). This behaviour was quite different from the increase in growth observed for these organisms. *Streptococcus thermophilus* and *L. delbrueckii* ssp. *bulgaricus* showed the best growth in RSM compared to the probiotics. The growth of probiotics is slow in milk because of their lower proteolytic activity (Klaver et al., 1993). *Streptococcus thermophilus* and *B. longum* showed significantly ($P < 0.05$) improved growth in RSMR1 than in RSM while *L. casei*, showed a slight, but nonsignificant, improvement. *Bifidobacterium longum* in particular showed nearly twice as much growth in RSMR1 than in RSM. This confirms the bifidogenic property of Raftiline HP which is a hot water extract from chicory roots with a DP > 23. Roberfroid et al. (1998) and Rossi et al. (2005) have also reported the bifidogenic nature of inulin and fructooligosaccharides. The ability of strains to grow in the presence of inulin is related to the production of extracellular enzymes that can hydrolyze long-chain fructans. Perrin et al. (2001) reported that most bifidobacteria possess inducible cell-associated β–fructofuranosides. However, *L. acidophilus* and *L. delbrueckii* ssp. *bulgaricus* showed a lower growth in RSMR1 than in RSM (Table 5.1). No significant ($P > 0.05$) changes in growth were observed for any of the organisms in RSMR2 while in RSMR3, except for *B. longum*, all others did not show any change in growth as compared to that in RSM. Özer et al. (2005) have reported that inulin did not stimulate the growth of *S. thermophilus*, *L. delbrueckii* ssp. *bulgaricus* and *L. acidophilus* but stimulated the growth of *B. bifidum*. Roberfroid et al. (1998) also found much lower growth rates for lactobacilli as compared to bifidobacteria in the presence of inulin. Shin et al. (2000) have indicated that the bifidogenic effect of inulin increased with increasing concentration of inulin. Thus, incorporation of Raftiline HP appears to support the growth of *B. longum* and *S. thermophilus* in particular. However, we observed that when *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* were co-cultured, the counts of *S. thermophilus* were lower in the presence of inulin while those of *L. delbrueckii* ssp. *bulgaricus* were higher (Ramchandran and Shah, 2008b). This indicates that the influence of inulin on growth of organisms also depended on the other organisms present. Thus, the effect of the varying concentrations of Raftiline HP on the growth of the different types of LAB differed with the type of organism (Table 5.1). It was also observed that growth of organisms does not necessarily translate in higher decreases in pH of the medium. Roberfroid et al. (1998) also observed that pH change is a relatively poor indicator of bacterial growth.

The counts of *S. thermophilus* 1275 and *L. delbrueckii* ssp. *bulgaricus* 1368, log CFU/g, in low-fat yogurts during storage at 4 °C for 28 days is presented in Table 5.2. The increase in the counts of *S. thermophilus* 1275 was significantly lower ($P < 0.05$) in the yogurts containing 2% Raftiline HP (YI2) than the control (YI0), but was similar in those containing 3% Raftiline HP (YI3). The increase in numbers of *L. delbrueckii* ssp. *bulgaricus* 1368 was higher ($P < 0.05$) in YI2 and YI3 as compared to the control (YI0). This indicates that Raftiline HP supported the growth of *L. delbrueckii* ssp. *bulgaricus* irrespective of the level of addition but did not help the growth of *S. thermophilus* as much, particularly at 2% level. During storage, there was a significant ($P < 0.05$) decline in the counts of *S. thermophilus* at day 7 of storage in YI0 while in YI2 and YI3 the decline was significant only on day 14, but thereafter, there was no decrease ($P > 0.05$) in their viability in all the three types of yogurt. At the end of storage (day 28) the delta log count of *S. thermophilus* was lowest in YI2 ($P < 0.05$) followed by YI0 and YI3 ($P > 0.05$), while the total decrease in log counts (day 1 minus day 28) was 0.27, 0.25 and 0.19 folds in YI0, YI2 and YI3 respectively. This confirms the stability of *S. thermophilus* during storage of yogurts, particularly in yogurt containing 3% Raftiline HP. On the other hand, *L. delbrueckii* ssp. *bulgaricus* exhibited a decline ($P < 0.05$) in numbers until day 21 in all the yogurts. A significantly higher number of *L. delbrueckii* ssp. *bulgaricus* were present in YI2 on day 28 followed by YI3 ($P > 0.05$) and YI0 ($P < 0.05$). The decrease in log counts at the end of storage (day 1 minus day 28) was 0.39 fold in YI0 while in YI2 and YI3 it was 0.3 fold. The accumulation of organic acids in yogurt during storage could have resulted in the reduction in numbers of the bacteria. Özer et al. (2005) have also reported that *S. thermophilus* was more stable than *L. delbrueckii* ssp. *bulgaricus* during storage of yogurts for 14 days at < 6 °C.

The reduction in pH during preparation of the three types of yogurts is presented in Table 5.3. The decrease in pH during the first three hours of fermentation was maximum in YI3 (1.37 units) followed by YI2 (1.29 units) and YI0 (1.14 units). Thereafter, the rate of decrease in pH was more in YI0 as compared to YI2 and YI3. However, yogurts containing Raftiline HP reached the pH of 4.5 earlier (by 10 min) than the control (YI0). Thus, incorporation of Raftiline HP appeared to improve the growth of starter organisms which resulted in shorter fermentation time. This is in agreement with the findings of Hardi and Slacanac (2000) as well as Özer et al. (2005), who reported that the rate of pH decrease of fermented milk products was increased by the addition of inulin. However, Robinson (1995)

did not find any difference in the rate of acid production by the starter cultures in yogurt milks containing inulin.

Changes in pH during storage of the three types of low-fat yogurts are exhibited in Figure 5.1. Incorporation of Raftiline HP did not have any influence ($P > 0.05$) on the changes in pH during storage of the low-fat yogurts. The decrease in pH was significant ($P < 0.05$) during the first 2 weeks of storage in all the three types of yogurt. Thereafter, there was very little change in pH ($P > 0.05$). However, there were differences in the pattern of change in pH during storage of YI0, YI2 and YI3. The drop in pH of YI0 was by 0.12 units, while that of YI2 and YI3 was 0.11 and 0.10 respectively, at day 7 of storage. Thereafter, at day 14 the drop in pH was lesser in YI0 (0.08 units) than in YI2 (0.14 units) and YI3 (0.13 units). All the batches reached nearly the same pH value (4.26-4.28) at the end of the storage (day 28). These changes in pH matched the decreases in the viability of the starter cultures (Table 5.2). The decrease in the viability of the starter cultures, particularly of *L. delbrueckii* ssp. *bulgaricus*, could have resulted in the slower decrease of pH towards the end of storage. These results are similar to those reported by Guven et al. (2005) who did not observe any influence of Raftiline HP on pH value of low-fat yogurt and found a decrease in pH during the 15 days of storage at 4 °C.

5.3.2 Organic acid production

The production of lactic and acetic acids (mg/mL) by the LAB in RSM with and without Raftiline HP (6 h values less the 0 h values) is shown in Table 5.4. *Streptococcus thermophilus* produced the maximum amount of lactic acid compared to the other LAB. This confirms the high adaptability of *S. thermophilus* to grow in a medium containing lactose. Among the probiotics, *B. longum* produced the maximum amount of lactic acid. Parche et al. (2006) have also found that *B. longum* NCC prefers to utilize lactose over other sugars via the bifid pathway. All organisms produced significantly ($P < 0.05$) higher amounts of lactic acid in RSMR1 than in RSM. Donkor et al. (2007d) have also noted increased lactic acid production, but not acetic acid, by strains of *L. acidophilus* and *L. casei* in the presence of inulin. Higher concentrations of Raftiline HP, however, did not result in significant ($P > 0.05$) increases in the concentration of lactic acid produced. On the other hand, a sharp decrease in the amount of lactic acid produced by *L. delbrueckii* ssp. *bulgaricus* was observed in RSMR2 and RSMR3. These patterns however do not match with either the growth or the pH changes observed for these organisms except for *B. longum* where the increased production of lactic and acetic acids matched with the increased growth of

B. longum in RSM containing Raftiline HP. The heterolactic nature of bifidobacteria (Oliveira et al., 2009b) could be responsible for the lower pH values in RSM containing Raftiline HP, despite the higher growth and acid production by *B. longum*. However, we have observed that when *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* were grown together in low-fat yogurt, the lactic acid and acetic acid concentrations decreased in the presence of inulin (Ramchandran and Shah, 2008b).

Acetic acid production in RSM was greatest by *S. thermophilus* (0.021 mg/mL) while all the other organisms produced between 0.018 to 0.019 mg/mL. There was a significant ($P < 0.05$) increase in the amount of acetic acid produced by all the organisms in RSMR1 with the exception of *S. thermophilus*. However, no further increase in the concentration of acetic acid produced was observed for any of the organisms grown in RSMR2 and RSMR3. Also, except for *S. thermophilus* and *B. longum*, all organisms tended to produce more acetic acid than lactic acid. Gardiner et al. (2002) have reported that bifidobacteria produce more acetic acid than lactic acid while for lactobacilli the reverse was true. Since the ratio of lactic to acetic acid influences the flavour balance in products such as yogurt (Chandan and O'Rell, 2006), the use of these organisms could influence the sensory characteristics of the product.

The concentration of lactic and acetic acids (mg/100g) in the three types of yogurts during storage at 4 °C for 28 days is presented in Table 5.5. On day 1 the amounts of lactic and acetic acid was more in YI0 ($P < 0.05$) than YI2 and YI3 and the concentration of both acids remained high ($P < 0.05$) until the end of storage. There was a significant ($P < 0.05$) increase in the concentration of lactic acid in all the three batches of yogurt on day 7 while YI0 continued to show an increase ($P < 0.05$) until day 14. On the other hand, there was no significant change ($P > 0.05$) in the concentration of acetic acid during storage of all the samples of yogurt, except for the decrease ($P < 0.05$) observed in YI0 sample on day 21. There was a decrease ($P > 0.05$) in the amount of acetic acid in YI2 and YI3 also, on day 21. Thus, incorporation of Raftiline HP did not result in increased concentration of lactic acid in the yogurts. The increase in concentration of lactic acid during the first 14 days of storage supported the decrease in pH observed during the same period (Figure 5.1)

5.3.3 Proteolytic activity

The proteolytic activity, measured as change in absorbance at 340 nm (A_{340}) between 0 h and 6 h samples, is represented in Figure 5.2. *Streptococcus thermophilus* and *L. delbrueckii* ssp. *bulgaricus* were the most proteolytic (A_{340} = 0.048-0.047) compared to

the probiotics (A_{340} = 0.003-0.024) in RSM. Among the probiotics, *L. casei* was the most proteolytic in RSM while *B. longum* was the least. Shihata and Shah (2000) have also reported comparatively high proteolytic activity for *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* and least for bifidobacteria. However, in the presence of Raftiline HP *L. acidophilus* and *B. longum* showed significantly ($P < 0.05$) increased proteolytic activity, particularly in RSMR2 for *B. longum*. Thereafter, while *L. acidophilus* showed a progressive increase in proteolysis in RSMR3, *B. longum* showed a slight decrease. *Lactobacillus casei*, *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus* showed lesser proteolytic tendencies in RSMR1 than in RSM, followed by a significant ($P < 0.05$) increase in proteolysis in RSMR2, except for *S. thermophilus* which continued to show a decrease ($P < 0.05$) in proteolysis. The changes in proteolysis do not relate to the changes in growth observed in RSM compared to RSMR1, RSMR2 and RSMR3, except for *B. longum* where high growth ($P < 0.05$) was observed in Raftiline HP supplemented RSM. So far no study has been conducted on the proteolytic capabilities of LAB in the presence of inulin. However, we have observed that the proteolytic activity of low-fat yogurt made with *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* was lower when 2% inulin was added but was higher in the presence of 3% inulin (Ramchandran and Shah, 2008b). It appears that incorporation of Raftiline HP, as well as the level of addition, had a varying influence on the proteolytic capabilities depending on the type of organism and that the influence can differ when the organisms were co-cultured.

The proteolytic activity of the starter cultures in the three types of yogurts stored at 4 °C for 28 days, measured as absorbance of free amino acids at 340 nm, is shown in Table 5.6. The proteolytic activity was maximal in YI3 throughout the storage period, being significant on day 1 and day 28. The extent of proteolysis on day 1 was highest in YI3 followed by YI0 and YI2. The increase in the amount of free amino acids continued throughout the storage period and was highest in YI3. The increases, however, were not significant until day 7 in case of YI0 and YI2 and until day 14 in case of YI3. Similarly, the increase in the amount of free amino acids in all three types of yogurt was not significant ($P > 0.05$) during the last week of storage. In an earlier work Ramchandran and Shah (2009b) found that incorporation of 2% Raftiline HP to RSM significantly improved the proteolytic capability of *L. delbrueckii* ssp. *bulgaricus* 1368 when grown individually. However, due to the complementary nature of the two organisms, improvement ($P < 0.05$) in the total proteolysis in the yogurt has been observed in the presence of Raftiline HP. So far there are no published reports on the extent of proteolysis in yogurt in the presence of inulin.

5.3.4 ACE-inhibition

Fermented milk containing strains of *L. delbrueckii* ssp *bulgaricus* and *S. thermophilus* has been reported to produce peptides with good ACE-inhibition potential (Gobbetti et al., 2000; Ashar and Chand, 2004; Korhonen and Pihlanto, 2006). Inhibition of ACE can exert antihypertensive effect (Korhonen and Pihlanto, 2006). The *in vitro* percent ACE-inhibition and the corresponding IC_{50} values of the TCA filtrates of the organisms studied are presented in Table 5.7. The 0 h TCA filtrates did not show any inhibition of ACE (data not shown). All the organisms showed good ACE-inhibition potential in RSM, with relatively low IC_{50} values. None of the organisms showed any significant increase in ACE-inhibition in RSMR1 compared to RSM with the exception of *L. casei*. *Streptococcus thermophilus* and *L. acidophilus* produced nearly the same percentage inhibition in RSM, RSMR1, RSMR2 and RSMR3. However, *L. delbrueckii* ssp. *bulgaricus* produced significantly ($P < 0.05$) higher percentage of ACE-inhibition in RMSR2 while for *B. longum* it was in RSMR3. These variations do not correlate with the proteolytic or growth pattern of these organisms. Pripp et al. (2006) have reported that inhibitory potencies of the peptides do not always correlate to the extent of proteolysis. There is no published literature available describing the effect of inulin addition on the ACE-inhibitory capabilities of LAB. Addition of Raftiline HP appears to boost the generation of ACE-inhibitory peptides by *L. casei*, *L. delbrueckii* ssp. *bulgaricus* and *B. longum* only. However, it has been reported that the ACE-inhibitory activity improved in low-fat yogurt made with 2 and 3% inulin (Ramchandran and Shah, 2008b).

The ACE-inhibition (%) and IC_{50} (mg/mL) of the filtrates prepared from the three batches of yogurts is given in Table 5.8. The ACE-inhibitory activity was highest in YI3 all through the storage of the yogurts, except on day 21 ($P > 0.05$). This is consistent with the highest proteolysis observed in YI3 (Table 5.6). Correspondingly, the IC_{50} (mg/mL) values of the YI3 filtrates were the lowest, indicating higher ACE-inhibitory activity as compared to YI2 and YI0. Fuglsang et al. (2003) have also observed that higher the amount of free amino groups, the higher the extent of ACE-inhibition in milks fermented by strains of *L. helveticus*. The ACE-inhibitory activity (%) of all the yogurts continued to increase during storage at 4 °C for 28 days. This increase was significant ($P < 0.05$) at day 7 and day 21 in YI0 and YI2, while for YI3 it was at day 14 and day 28. This indicated that during storage, with the continued proteolysis (Table 5.6), several potent peptides were being generated in the presence of Raftiline HP that showed good ACE-inhibition activity. A good correlation

was observed between proteolysis and ACE-inhibition for all the yogurts (r = 0.99 for YI0 and YI2, r = 0.80 for YI3).

5.3.5 α-Glu inhibition

The control of postprandial hyperglycemia is critical in the early therapy for diabetes. One therapeutic approach to decrease postprandial hyperglycemia is to retard the absorption of glucose by inhibiting enzymes such as α-glu (Kim et al., 2005). Much of the work related to α-glu inhibition has involved the use of plant extracts and some traditional foods (Fujita et al., 2003; Djomeni et al., 2006). Limited work has been conducted on the anti-diabetic potential of LAB (Yadav et al., 2007). The TCA filtrates of the organisms selected for this experiment showed good α-glu inhibition with low IC_{50} values (Table 5.9). Among the yogurt starters grown in RSM, *L. delbrueckii* ssp. *bulgaricus* was more inhibitive towards the activity of α-glu than *S. thermophilus* while among the probiotics *B. longum* was the most inhibitive. All organisms produced higher percent inhibition of α-glu in the presence of Raftiline HP than when grown in RSM with the exceptions of *L. delbrueckii* ssp. *bulgaricus* at all concentrations of Raftiline HP and *L. casei* and *L. acidophilus* in RSMR3 and RSMR2 respectively. *Lactobacillus acidophilus* and *S. thermophilus* exhibited highest inhibition in RSMR3; *B. longum* and *L. casei* exhibited maximum inhibition in RSMR1 while *L. delbrueckii* ssp. *bulgaricus* exhibited maximum inhibition in RSM. This indicates LAB could be capable of inhibiting α-glu activity which could make fermented milk a potential anti-diabetic food. Also addition of Raftiline HP at different concentrations had varying influence among the different species of LAB.

5.3.6 Whey separation

Table 5.10 shows the spontaneous whey separation (%) in all the three types of low-fat yogurts during storage at 4 °C for 28 days. Incorporation of Raftiline HP did not affect ($P > 0.05$) the whey separation throughout the storage period. However, there were differences in the pattern of whey separation during storage. Yogurts containing Raftiline HP showed a decrease ($P > 0.05$) in whey separation on day 7 followed by an increase ($P < 0.05$ for YI3 and $P > 0.05$ for YI2) during the rest of the storage period. On the other hand, YI0 did not exhibit any change on day 7 but showed a decrease ($P < 0.05$) at day 14 followed by an increase ($P < 0.05$) at day 28. These observations were similar to those observed by Guven et al. (2005), who concluded that inulin did not affect whey separation in yogurt and that whey separation reduced significantly during 15 days of storage at 4 °C. Contrarily,

Aryana and McGrew (2007) found that inulin HP having a longer chain length had significantly lower syneresis while Ipsen et al. (2001) reported that increasing the amount of inulin in yogurt manufacture caused coarser acid-induced protein network that resulted in increased syneresis. Ibrahim et al. (2004) observed a decrease in syneresis during storage while Dello Staffolo et al. (2004) reported that yogurt containing inulin did not undergo syneresis during storage. Seydm et al. (2005) did not observe any changes in syneresis in low-fat yogurt during 14 days storage. Variations in the reports regarding whey separation/syneresis may be due to differences in analysis methods and also in conditions of manufacture and storage of yogurts.

5.3.7 Firmness

The firmness (g) of the three low-fat yogurts during storage at 4 °C for 28 days is presented in Table 5.11. There was no significant ($P > 0.05$) difference in the firmness among the three types of yogurt throughout the storage period. All yogurts exhibited an increase in firmness during storage being significant at day 7 for YI0 and YI3, and at day 7 and 14 for YI2. At the end of storage, all the yogurts had nearly the same level (81.61-82.90 g) of firmness. In contrast, Bozanic et al. (2001) found that the firmness of yogurt improved upon addition of inulin. It has been speculated that since inulin is a water-structuring agent, it can complex with protein aggregates in yogurt and may cause increased firmness (Kip et al., 2006). However, Robinson (1995) hypothesised that oligosaccharides such as inulin caused less serious interference with the degree of protein-protein bonding even at 10% level of inclusion and that beyond 5% level of inclusion, the gel could be disintegrated into smaller fragments and hence the overall impact of inulin on the firmness is very small.

5.3.8 Viscoelastic properties of fresh yogurts

The viscoelastic properties of the three types of fresh low-fat yogurt were assessed using a small amplitude oscillatory measurement and results are presented in Figure 5.3. The flow behaviour of the samples under increasing shear rate was modelled using the Power law and Herschel-Bulkley models. The coefficients associated with these two models are presented in Table 5.12. All samples showed a response typical of weak gels with storage modulus (G') higher than loss modulus (G''). Addition of Raftiline HP resulted in a decrease in G' values (Figure 5.3) which indicates that Raftiline HP imparted less solid-like (elastic) properties to the low-fat yogurt. The decrease was more upon 2% addition of Raftiline HP (YI2) with the maximum of 52 Pa as opposed to 3% (YI3) addition and control (YI0) with

60.22 and 68.62 Pa, respectively (Figure 5.3). Similarly, tan δ values for YI2 sample was the lowest followed by YI3 and YI0, another indicator of less solid like properties in yogurts containing Raftiline HP. According to Kim et al. (2001), heating inulin solutions to temperatures above 70 °C causes degradation of Raftiline HP into shorter chains by hydrolysis, which makes it harder to form a gel. Thus, heating of mixes with Raftiline HP to 85 °C for 30 min could have resulted in hydrolysis of Raftiline HP which could be the reason for less elastic and more plastic like properties observed in the resulting yogurts. Patocka et al. (2006) also found that decrease in the G′ values in yogurt could be rationalized in terms of network weakening. The yield stress also decreased in yogurts containing Raftiline HP (Table 5.12) which was in concurrence with the decrease in G′ values of the samples (Figure 5.3). Furthermore, the consistency index as estimated by the Power law decreased ($P > 0.05$) in yogurts containing Raftiline HP, being lower ($P > 0.05$) in YI2 than in YI3 while the control yogurts showed highest consistency (Table 5.12). These results indicate that the addition of Raftiline HP could adversely affect the consistency of the products in comparison to the control. These products were more fluid like with distinct pseudoplastic properties and lesser ability to resist deformation upon applied shear. On the contrary, Guven et al. (2005) observed that while the consistency of yogurt containing 1% inulin was lower, those containing 3% inulin was higher than control while those containing 2% inulin exhibited highest consistency.

5.4 Conclusion

This study confirms the bifidogenic nature of Raftiline HP as it significantly improved the growth of *B. longum* and also improved the growth of *S. thermophilus* in RSM. All organisms produced more lactic acid when grown in the presence of 1% Raftiline HP but there was no effect on reduction of pH of the medium with the exception of *B. longum* and *L. delbrueckii* ssp. *bulgaricus*. Incorporation of Raftiline HP, as well as the level of addition, had a varying influence on the proteolytic capabilities depending on the type of organism while the generation of ACE-inhibitory peptides by *L. casei*, *L. delbrueckii* ssp. *bulgaricus* and *B. longum* only was improved. However, there was an improvement in the α-glu inhibitory activities of all the organisms in RSMR3, except that of *L. delbrueckii* ssp. *bulgaricus* and *L. casei.* All organisms showed lower α-glu inhibitory activities in RSMR2 than in RMSR1. The level of addition of Raftiline HP showed varying influences on the

growth and biochemical activities of the organisms. Thus, it appears that Raftiline HP not only enhanced the growth of *B. longum* but also improved some of its biochemical activities. Such a study can be useful in deciding the level of Raftiline HP required to be added for improved bioactivity of the organisms used in food fermentations.

Incorporation of Raftiline HP in low-fat yogurts appeared to improve the growth of starter organisms which resulted in shorter fermentation time. Raftiline HP supported the growth of *L. delbrueckii* ssp. *bulgaricus* irrespective of the level of addition but did not help the growth of *S. thermophilus* as much, particularly at 2% level. However, there were no significant changes in pH during storage nor any increase in concentration of lactic acid in the low-fat yogurts containing Raftiline HP compared to control. Improvement in total proteolysis in the yogurts was observed in the presence of Raftiline HP and was highest in yogurt containing 3% Raftiline HP. Consequently, the ACE-inhibitory activity was maximal in YI3 all through the storage of the yogurts, indicating better ACE-inhibitory activity as compared to YI2 and YI0. Incorporation of Raftiline HP did not affect the textural properties of the low-fat yogurts such as whey separation and firmness. The products were more fluid like than the control with distinct pseudoplastic properties and lesser ability to resist deformation upon applied shear.

Table 5.1 Changes in growth and pH values of selected lactic acid bacteria (LAB) and *Bifidobacterium* grown in reconstituted skim milk (RSM) with and without Raftiline HP at 37 ºC for 6 h.

LAB	Delta pH values				Delta log_{10} CFU/mL			
	RSM	RSMR1	RSMR2	RSMR3	RSM	RSMR1	RSMR2	RSMR3
LC	0.11 ± 0.02^{a}	0.11 ± 0.01^{a}	0.15 ± 0.01^{b}	0.15 ± 0.01^{a}	0.86 ± 0.04^{a}	0.95 ± 0.15^{a}	0.93 ± 0.12^{a}	0.80 ± 0.05^{ab}
LA	0.11 ± 0.00^{a}	0.08 ± 0.01^{ab}	0.09 ± 0.01^{a}	0.10 ± 0.01^{a}	0.46 ± 0.13^{a}	0.38 ± 0.14^{a}	0.41 ± 0.07^{a}	0.40 ± 0.07^{a}
ST	1.06 ± 0.00^{a}	1.06 ± 0.00^{a}	1.07 ± 0.01^{ab}	1.08 ± 0.01^{b}	1.96 ± 0.10^{a}	2.12 ± 0.08^{b}	2.03 ± 0.03^{abc}	2.01 ± 0.03^{ac}
LB	0.36 ± 0.02^{a}	0.18 ± 0.01^{b}	0.19 ± 0.01^{b}	0.18 ± 0.01^{b}	1.22 ± 0.07^{a}	1.19 ± 0.03^{a}	1.16 ± 0.06^{a}	1.29 ± 0.06^{ab}
BL	0.13 ± 0.01^{a}	0.09 ± 0.01^{b}	0.10 ± 0.01^{b}	0.09 ± 0.01^{b}	0.33 ± 0.11^{a}	0.54 ± 0.07^{b}	0.59 ± 0.08^{b}	0.61 ± 0.11^{b}

Results presented are means ± SEM of six observations, P = 0.05.
RSMR1 is RSM containing 1% Raftiline HP, RSMR2 is RSM containing 2% Raftiline HP, RSMR3 is RSM containing 3% Raftiline HP
[ab]Means in the same row of each parameter with different lowercase letters are significantly (P < 0.05) different.
LC = *L. casei* 15286, LA = *L. acidophilus* 4461, ST = *S. thermophilus* 1275, LB = *L. delbrueckii* ssp. *bulgaricus* 1368, BL = *B. longum* 5022

Table 5.2 Counts (Δlog CFU/g) of *S. thermophilus* 1275 and *L. delbrueckii* ssp. *bulgaricus* 1368 in yogurts with or without Raftiline HP stored at 4 °C for 28 d.

	Storage Period				
	Day 1	Day 7	Day 14	Day 21	Day 28
S. thermophilus					
YI0	2.32^{aA}	2.11^{bA}	2.11^{bA}	2.10^{bA}	2.05^{bA}
YI2	2.21^{aB}	2.14^{aA}	2.04^{bA}	1.97^{bB}	1.96^{bB}
YI3	2.30^{aAB}	2.22^{aBA}	2.09^{bA}	2.09^{bA}	2.11^{bA}
SEM			0.04		
L. delbrueckii ssp. *bulgaricus*					
YI0	2.17^{aA}	1.98^{bA}	1.98^{bA}	1.78^{cA}	1.78^{cA}
YI2	2.29^{aB}	2.18^{bB}	2.08^{cB}	2.03^{cdB}	1.99^{dB}
YI3	2.23^{aAB}	2.08^{bC}	2.02^{bAB}	1.94^{cbC}	1.93^{cB}
SEM			0.03		

Values are the statistical means less the initial count at 0 h.
SEM = standard error of means
YI0 = control yogurt prepared from skim milk standardized to 12% total solids; YI2 = yogurt prepared from skim milk standardized to 12% total solids and added with 2% (w/v) Raftiline HP; YI3 = yogurt prepared from skim milk standardized to 12% total solids and added with 3% (w/v) Raftiline HP
^{abcd}Means in the same row with different alphabets are significantly different within a particular treatment for each organism
^{ABC}Means in the same column with different alphabets are significantly different for a particular day of storage for each organism

Table 5.3 Changes in pH of yogurt mixes with or without Raftiline HP during preparation of low-fat yogurt at 42 °C.

Period of incubation	Type of yogurt		
	YI0	YI2	YI3
0 h	6.46	6.45	6.43
3 h	5.33	5.15	5.06
3 h 30 min	5.02	4.96	4.84
4 h	4.68	4.66	4.58
4 h 30 min	4.52	4.50	4.50
4 h 40 min	4.46	-	-

Values presented are average of three replicates
YI0 = control yogurt prepared from skim milk standardized to 12% total solids; YI2 = yogurt prepared from skim milk standardized to 12% total solids and added with 2% (w/v) Raftiline HP; YI3 = yogurt prepared from skim milk standardized to 12% total solids and added with 3% (w/v) Raftiline HP

Table 5.4 Concentration of lactic and acetic acid (mg/mL) produced by selected lactic acid bacteria (LAB) and *Bifidobacterium* grown in reconstituted skim milk (RSM) with and without Raftiline HP at 37 ºC for 6 h.

LAB	Lactic acid				Acetic acid			
	RSM	RSMR1	RSMR2	RSMR3	RSM	RSMR1	RSMR2	RSMR3
LC	0.016 ± 0.002^{a}	0.021 ± 0.001^{b}	0.023 ± 0.001^{bc}	0.024 ± 0.000^{bc}	0.018 ± 0.002^{a}	0.021 ± 0.000^{b}	0.023 ± 0.001^{bc}	0.023 ± 0.000^{bc}
LA	0.011 ± 0.003^{a}	0.023 ± 0.001^{b}	0.021 ± 0.001^{b}	0.021 ± 0.000^{b}	0.019 ± 0.001^{a}	0.023 ± 0.001^{b}	0.022 ± 0.000^{bc}	0.023 ± 0.000^{b}
ST	0.041 ± 0.000^{a}	0.048 ± 0.002^{b}	0.046 ± 0.001^{ab}	0.036 ± 0.006^{bc}	0.021 ± 0.001^{a}	0.020 ± 0.001^{a}	0.020 ± 0.001^{a}	0.022 ± 0.002^{a}
LB	0.011 ± 0.001^{a}	0.020 ± 0.002^{b}	0.009 ± 0.000^{ac}	0.009 ± 0.000^{bc}	0.019 ± 0.000^{a}	0.021 ± 0.000^{b}	0.022 ± 0.001^{b}	0.022 ± 0.001^{b}
BL	0.017 ± 0.000^{a}	0.039 ± 0.001^{b}	0.039 ± 0.001^{b}	0.038 ± 0.001^{b}	0.019 ± 0.001^{a}	0.024 ± 0.001^{b}	0.024 ± 0.001^{b}	0.025 ± 0.001^{b}

Results presented are means ± SEM of six observations, P = 0.05
RSMR1 is RSM containing 1% Raftiline HP, RSMR2 is RSM containing 2% Raftiline HP, RSMR3 is RSM containing 3% Raftiline HP
[abc]Means in the same row of each parameter with different lowercase letters are significantly (P < 0.05) different.
LC = *L. casei* 15286, LA = *L. acidophilus* 4461, ST = *S. thermophilus* 1275, LB = *L. delbrueckii* ssp. *bulgaricus* 1368, BL = *B. longum* 5022

Table 5.5 Concentration of lactic and acetic acids (mg/100g) in low-fat yogurts with or without Raftiline HP stored at 4 °C for 28 d.

	Storage Period				
	Day 1	Day 7	Day 14	Day 21	Day 28
Lactic acid					
YI0	1.15^{aA}	1.23^{bA}	1.29^{cA}	1.22^{bA}	1.31^{cA}
YI2	1.06^{aB}	1.14^{bB}	1.23^{bcAB}	1.17^{bA}	1.22^{bcB}
YI3	1.10^{aB}	1.14^{aB}	1.19^{baB}	1.21^{bA}	1.22^{bB}
SEM			0.08		
Acetic acid					
YI0	0.07^{aA}	0.10^{aA}	0.10^{aA}	0.03^{bA}	0.10^{caA}
YI2	0.02^{aB}	0.05^{aB}	0.03^{aB}	ND	0.04^{aB}
YI3	0.03^{aB}	0.03^{aB}	0.04^{aC}	0.04^{aA}	0.04^{aB}
SEM			0.06		

Values are the statistical means of six observations
SEM = standard Error of Means
YI0 = control yogurt prepared from skim milk standardized to 12% total solids; YI2 = yogurt prepared from skim milk standardized to 12% total solids and added with 2% (w/v) Raftiline HP; YI3 = yogurt prepared from skim milk standardized to 12% total solids and added with 3% (w/v) Raftiline HP
^{abc}Means in the same row with different alphabets are significantly different within a particular treatment for each organic acid
^{ABC}Means in the same column with different alphabets are significantly different for a particular day of storage for each organic acid

Table 5.6 Proteolysis (Δ absorbance at 340 nm) in low-fat yogurts with or without Raftiline HP stored at 4 °C for 28 d.

	Storage Period				
	Day 1	Day 7	Day 14	Day 21	Day 28
YI0	0.245^{aA}	0.342^{aA}	0.394^{baA}	0.665^{cA}	0.649^{cA}
YI2	0.233^{aA}	0.352^{aAB}	0.410^{baA}	0.719^{cA}	0.680^{cA}
YI3	0.382^{aB}	0.412^{aB}	0.481^{aA}	0.762^{bA}	0.784^{bB}
SEM			0.038		

Values are the statistical means of six observations less the initial absorbance values at 0 h
SEM = standard error of means
YI0 = control yogurt prepared from skim milk standardized to 12% total solids; YI2 = yogurt prepared from skim milk standardized to 12% total solids and added with 2% (w/v) Raftiline HP; YI3 = yogurt prepared from skim milk standardized to 12% total solids and added with 3% (w/v) Raftiline HP
^{abc}Means in the same row with different alphabets are significantly different within a particular treatment
^{AB}Means in the same column with different alphabets are significantly different for a particular day of storage

Table 5.7 Effect of addition of Raftiline HP on angiotensin-I converting enzyme-inhibition (ACEI) ability and IC_{50} values of selected lactic acid bacteria and *Bifidobacterium* grown in reconstituted skim milk (RSM) at 37 ºC for 6 h.

LAB	RSM		RSMR1		RSMR2		RSMR3	
	ACEI (%)	IC_{50} (mg/mL)	ACEI (%)	IC_{50} (mg/mL)	ACEI (%)	IC_{50} (mg/mL)	ACEI (%)	IC_{50} (mg/mL)
L. casei 15286	25.21 ± 2.40^{a}	0.38	36.66 ± 3.41^{b}	0.71	39.38 ± 2.95^{b}	0.68	30.51 ± 3.03^{bc}	0.96
L. acidophilus 4461	39.32 ± 2.44^{a}	0.34	35.85 ± 3.92^{a}	0.75	36.51 ± 2.71^{a}	0.81	36.12 ± 2.56^{a}	0.79
S. thermophilus 1275	38.20 ± 0.66^{a}	0.27	37.58 ± 3.23^{a}	0.62	36.89 ± 2.23^{a}	0.73	36.97 ± 3.20^{a}	0.70
L. delbrueckii ssp. *bulgaricus* 1368	40.39 ± 4.01^{a}	0.36	42.45 ± 3.32^{a}	0.38	70.72 ± 2.33^{b}	0.31	37.67 ± 2.71^{a}	0.65
B. longum 5022	41.39 ± 3.53^{a}	0.30	32.96 ± 2.07^{b}	0.59	33.83 ± 2.17^{b}	0.67	47.09 ± 2.18^{bc}	0.47

Results presented are means ± SEM of six observations, P = 0.05.
RSMR1 is RSM containing 1% Raftiline HP, RSMR2 is RSM containing 2% Raftiline HP, RSMR3 is RSM containing 3% Raftiline HP
[abc]Means in the same row with different lowercase letters are significantly ($P < 0.05$) different.

Table 5.8 Changes in ACE-inhibition (%) and corresponding IC_{50} (mg/mL)in low-fat yogurts with or without Raftiline HP stored at 4 °C for 28 d.

	Storage Period									
	Day 1		Day 7		Day 14		Day 21		Day 28	
	ACEI	IC_{50}	ACEI	IC_{50}	ACEI	IC_{50}	ACEI	IC_{50}	ACEI	IC_{50}
YI0	7.42^{aA}	0.04	17.82^{bA}	0.02	22.53^{bA}	0.02	37.93^{cA}	0.01	40.20^{cA}	0.01
YI2	15.25^{aB}	0.02	22.24^{bA}	0.02	26.04^{bA}	0.01	39.75^{cA}	0.01	38.72^{cA}	0.01
YI3	20.94^{aC}	0.01	24.59^{aA}	0.01	39.94^{bB}	0.01	35.95^{bA}	0.01	58.15^{cB}	0.01
SEM	1.77									

Values are the statistical means of six observations
SEM = standard error of means
YI0 = control yogurt prepared from skim milk standardized to 12% total solids; YI2 = yogurt prepared from skim milk standardized to 12% total solids and added with 2% (w/v) Raftiline HP; YI3 = yogurt prepared from skim milk standardized to 12% total solids and added with 3% (w/v) Raftiline HP
^{abc}Means in the same row with different alphabets are significantly different within a particular treatment
^{ABC}Means in the same column with different alphabets are significantly different for a particular day of storage

Table 5.9 Effect of addition of Raftiline HP on alpha-glucosidase (α-Glu) inhibition ability and IC_{50} values of selected lactic acid bacteria and *Bifidobacterium* grown in reconstituted skim milk (RSM) at 37 ºC for 6 h.

LAB	RSM		RSMR1		RSMR2		RSMR3	
	α-Glu inhibition (%)	IC_{50} (mg/mL)	α-Glu inhibition (%)	IC_{50} (mg/mL)	α-Glu inhibition (%)	IC_{50} (mg/mL)	α-Glu inhibition (%)	IC_{50} (mg/mL)
L. casei 15286	62.29 ± 1.45^{a}	0.15	75.80 ± 2.88^{b}	0.34	72.07 ± 3.92^{b}	0.37	56.50 ± 2.38^{bc}	0.52
L. acidophilus 4461	63.83 ± 1.49^{a}	0.21	67.37 ± 2.28^{a}	0.40	61.07 ± 1.57^{ac}	0.49	88.58 ± 3.42^{bc}	0.32
S. thermophilus 1275	65.84 ± 2.08^{a}	0.16	70.28 ± 1.56^{b}	0.33	67.07 ± 0.98^{a}	0.40	76.71 ± 1.73^{bc}	0.34
L. delbrueckii ssp. *bulgaricus* 1368	73.45 ± 3.45^{a}	0.20	71.82 ± 3.56^{ac}	0.23	67.77 ± 2.12^{bc}	0.32	68.69 ± 0.57^{ac}	0.36
B. longum 5022	67.55 ± 2.54^{a}	0.18	81.77 ± 1.61^{b}	0.24	73.98 ± 2.14^{bc}	0.31	75.49 ± 3.78^{bc}	0.29

Results presented are means ± SEM of six observations, P = 0.05.
RSMR1 is RSM containing 1% Raftiline HP, RSMR2 is RSM containing 2% Raftiline HP, RSMR3 is RSM containing 3% Raftiline HP
^{abc}Means in the same row with different lowercase letters are significantly (P<0.05) different.

Table 5.10 Spontaneous whey separation (%) in low-fat yogurts with or without Raftiline HP stored at 4 °C for 28 d.

	Storage Period			
	Day 1	Day 7	Day 14	Day 28
YI0	2.53^{aA}	2.59^{aA}	1.74^{bA}	2.63^{aA}
YI2	2.61^{aA}	2.09^{aA}	2.38^{aAB}	2.55^{aA}
YI3	2.54^{aA}	2.07^{aA}	2.84^{abB}	2.91^{abA}
SEM	0.25			

Values are the statistical means of six observations
SEM = standard error of means
YI0 = control yogurt prepared from skim milk standardized to 12% total solids; YI2 = yogurt prepared from skim milk standardized to 12% total solids and added with 2% (w/v) Raftiline HP; YI3 = yogurt prepared from skim milk standardized to 12% total solids and added with 3% (w/v) Raftiline HP
[ab]Means in the same row with different alphabets are significantly different within a particular treatment
[AB]Means in the same column with different alphabets are significantly different for a particular day of storage

Table 5.11 Firmness (g) of low-fat yogurts prepared with or without Raftiline HP during 28 d cold (4 °C) storage.

	Storage Period			
	Day 1	Day 7	Day 14	Day 28
YI0	68.01^{aA}	76.62^{bA}	80.03^{bA}	81.61^{bA}
YI2	71.92^{aA}	77.54^{bA}	83.24^{cA}	82.90^{cA}
YI3	68.08^{aA}	76.99^{bA}	81.65^{bA}	82.18^{abA}
SEM	2.01			

Values are the statistical means of six observations at 95% level of confidence
SEM = standard error of means
YI0 = control yogurt prepared from skim milk standardized to 12% total solids; YI2 = yogurt prepared from skim milk standardized to 12% total solids and added with 2% (w/v) Raftiline HP; YI3 = yogurt prepared from skim milk standardized to 12% total solids and added with 3% (w/v) Raftiline HP
[ab]Means in the same row with different alphabets are significantly different within a particular treatment
[A]Means in the same column with similar alphabets do not differ significantly for a particular day of storage

Table 5.12 Flow behaviour of fresh low-fat yogurts prepared by addition of 2 or 3% Raftiline HP predicted by the Ostwald (Power law) and Herschel-Bulkley models.

	Power law model			Herschel-Bulkley model			
	k (Pa/s)	n	R^2	σ_0 (Pa)	k (Pa/s)	n	R^2
YI0	5.13[a]	0.415[a]	0.988	4.76[a]	1.85[a]	0.633[a]	0.988
YI2	4.01[a]	0.425[a]	0.987	3.51[a]	2.03[a]	0.592[a]	0.987
YI3	4.51[a]	0.437[a]	0.988	3.00[a]	2.01[a]	0.612[a]	0.985
SEM	0.47	0.007		0.57	0.14	0.029	

Values are the statistical means of six observations at 95% level of confidence
SEM = standard error of means
YI0 = control yogurt prepared from skim milk standardized to 12% total solids; YI2 = yogurt prepared from skim milk standardized to 12% total solids and added with 2% (w/v) Raftiline HP; YI3 = yogurt prepared from skim milk standardized to 12% total solids and added with 3% (w/v) Raftiline HP
[a]Means in the same column with similar alphabets do not differ significantly within a particular parameter

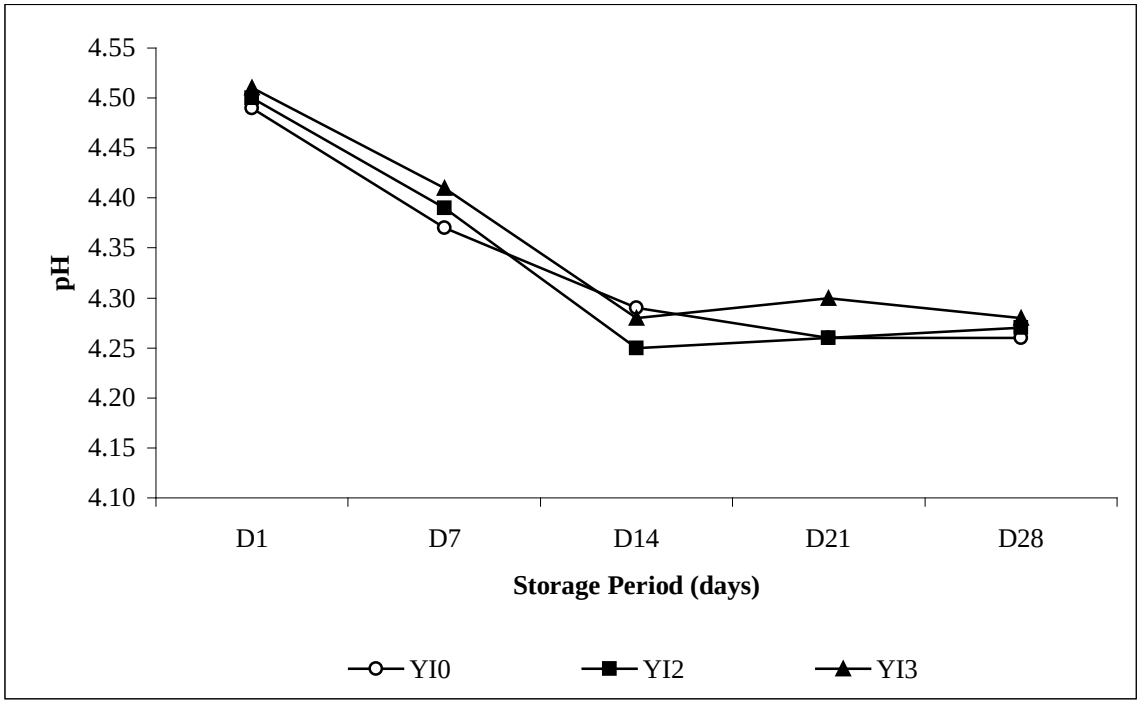

Figure 5.1 pH of low-fat yogurts with or without Raftiline HP and changes during storage at 4 °C for 28 d. YI0 = control yogurt prepared from skim milk standardized to 12% total solids; YI2 = yogurt prepared from skim milk standardized to 12% total solids and added with 2% (w/v) Raftiline HP; YI3 = yogurt prepared from skim milk standardized to 12% total solids and added with 3% (w/v) Raftiline HP.

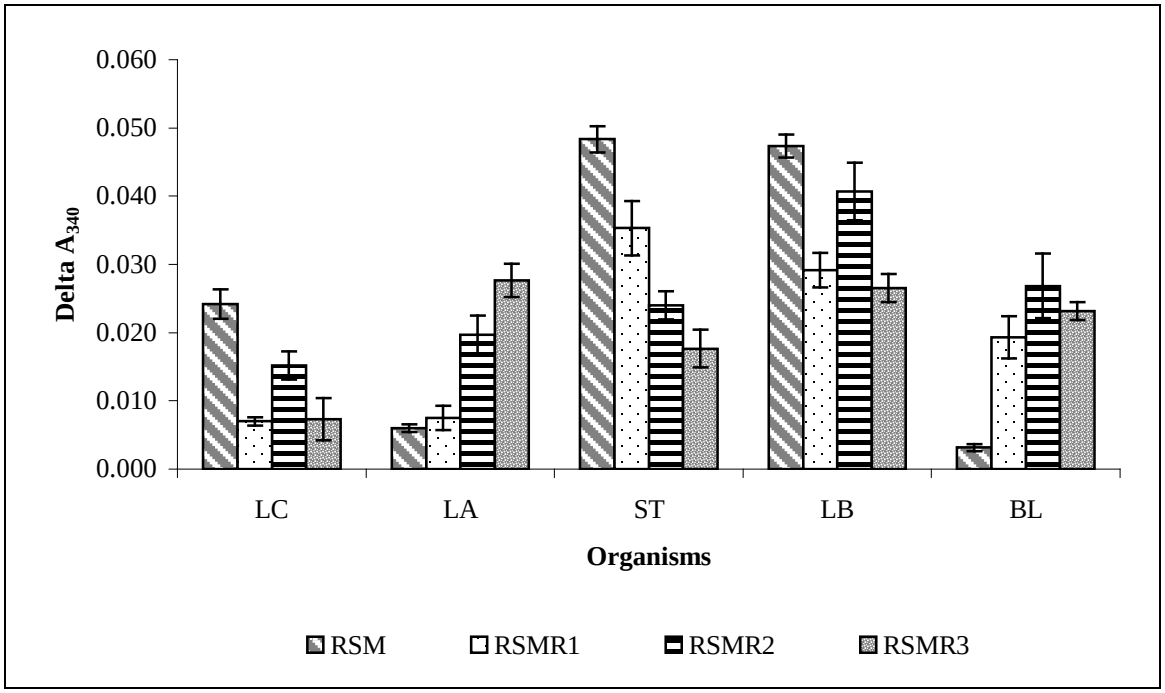

Figure 5.2 Changes in proteolytic capability (delta A_{340}) of selected lactic acid bacteria grown in reconstituted skim milk (RSM) with and without Raftiline HP, at 37 °C for 6 h. RSMR1 is RSM containing 1% Raftiline HP (bars with black dots); RSMR2 is RSM containing 2% Raftiline HP (bars with horizontal lines); RSMR3 is RSM containing 3% Raftiline HP (bars with black and white pattern). Bars with slanted lines is for RSM. LC = *L. casei* 15286, LA = *L. acidophilus* 4461, ST = *S. thermophilus* 1275, LB = *L. delbrueckii* ssp. *bulgaricus* 1368, BL = *B. longum* 5022 (Error bars represent the standard error of the means)

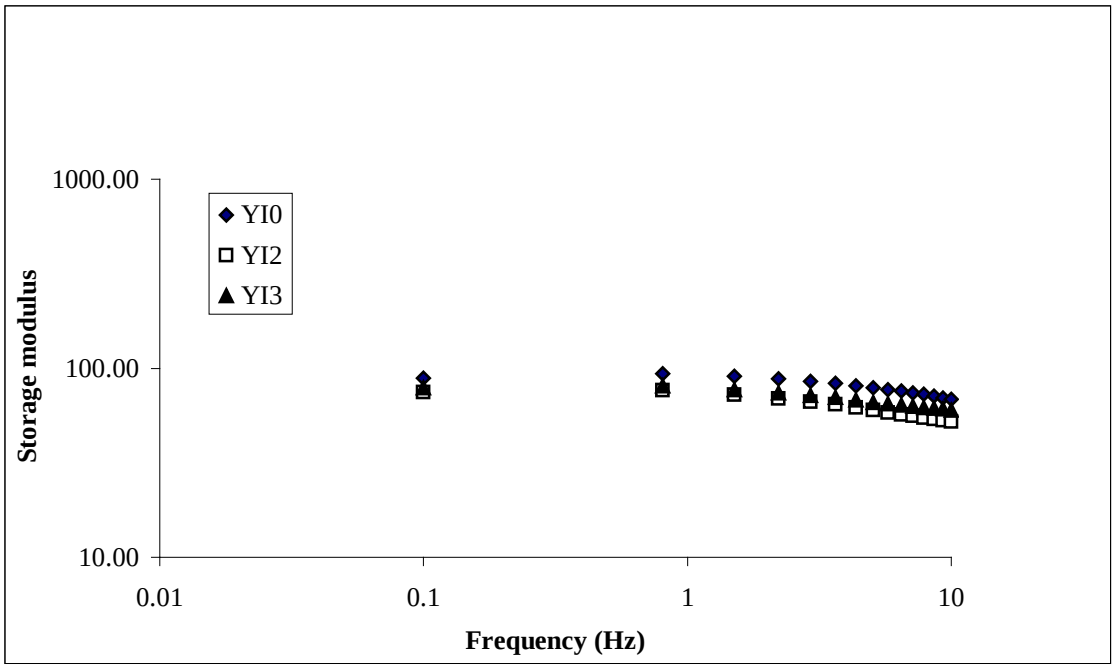

Figure 5.3 Elastic properties (G′) of low-fat yogurts prepared with or without Raftiline HP after manufacturing. YI0 = control yogurt prepared from skim milk standardized to 12% total solids; YI2 = yogurt prepared from skim milk standardized to 12% total solids and added with 2% (w/v) Raftiline HP; YI3 = yogurt prepared from skim milk standardized to 12% total solids and added with 3% (w/v) Raftiline HP.

6.0 Effect of Exopolysaccharides on the Proteolytic and ACE-Inhibitory Activities and Textural and Rheological Properties of Low-fat Yogurt, Inulin Containing Low-fat Yogurt and Inulin Containing Probiotic Yogurt During Refrigerated Storage

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6.1 Introduction

Exopolysaccharides (EPS) are long chain polysaccharides consisting of branched, repeating units of sugars or sugar derivatives. Lactic acid bacteria (LAB) are able to produce several types of polysaccharides that are classified according to their location relative to the cell. Those that are excreted outside the cell wall are called exocellular polysaccharides or EPS whereas those that form adherent cohesive layers are called capsular polysaccharides. The EPS can either be loosely attached or completely excreted into the medium as a slime (Ruas-Madiedo et al., 2002). Exopolysaccharides from LAB have proved to be invaluable in their application in the improvement of rheology, texture and mouthfeel of fermented milk products such as yogurt, particularly in low-fat yogurts. Exopolysaccharides produced by LAB are reported to improve the texture and firmness and reduce thermal and physical shock and syneresis and increase the viscosity of yogurt (DeVuyst and Degeest, 1999; Hugenholtz and Smid, 2002; Jolly et al., 2002; Ruas-Madiedo et al., 2002; Broadbent et al., 2003; Welman and Maddox, 2003; Sodini et al., 2004). The EPS produced by LAB are also reported to provide physiological benefits such as cholesterol lowering property, immunomodulation and antitumour activity (Hugenholtz and Smid, 2002; Welman and Maddox, 2003).

Certain strains of both the yogurt starter cultures, namely *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus*, are known to produce heteropolysaccharides. The quantity of EPS ranges from 50 to 350 mg/L for *S. thermophilus* and 60 to 150 mg/L for *L. delbrueckii* ssp. *bulgaricus* (Ruas-Madiedo et al., 2002). Although these organisms produce EPS at low levels, they still contribute to the texture, mouthfeel, taste perception and stability of the final products (Doleyres et al., 2005). Growth conditions, type of nutrients available and the strain of the organism are among the factors that influence the yield and type of EPS produced. In most industrial fermentations, EPS producing strains of *S. thermophilus* is the rational choice for products like yogurt mainly due to its minor role in proteolysis during milk fermentation (De Vuyst et al., 2003).

Yogurt already has an established image as a healthy food. The consumption of yogurt to maintain good health is a long tradition in many countries. It is now established that several peptide sequences are encrypted in milk proteins that have the potential to provide several physiological and health benefits to the consumer. These peptides are referred as bioactive peptides (Clare and Swaisgood, 2000; Shah, 2000b). Milk fermentations using LAB release these peptides (Meisel and Bockelmann, 1999; Gobbetti et al., 2002) which are reported to provide several health benefits. Peptides that provide

antihypertensive effects have caught the interest of scientists, manufacturers and consumers. These peptides are understood to have the potential to inhibit the activity of angiotensin-I converting enzyme (ACE) and thereby regulate blood pressure. Several ACE-inhibitory peptides have been isolated from fermented milk and commercial dairy products (Shah, 2000b; López-Fandiño et al., 2006; Hayes et al., 2007).

Fermented milks such as yogurt have been reported to provide a range of beneficial properties to humans, including assimilation of cholesterol, anti-tumorgenic effect, prevention of gastrointestinal infection, and lowering of blood pressure. During fermentation, LAB produce a range of secondary metabolites, some of which have been associated with health-promoting properties. ACE-inhibitory peptides produced during fermentation of milk are already the basis of health claims associated with some functional foods on the market such as Calpis and Evolus (Stanton et al., 2005). Proteolytic strains of probiotic bacteria are preferably used to release bioactive peptides, such as ACE-inhibitory peptides, for further improving the health benefits of probiotic foods such as yogurt (Shah, 2007). The blood pressure lowering effects in such products are mediated through the inhibition of angiotensin-I-converting-enzyme (ACE). Many of the peptides released from milk proteins due to the action of cell wall associated proteinase activity during fermentation have been found to possess such an inhibitory property. Since these proteolytic enzymes are not very specific, a great variety of peptides are liberated during fermentation (Fuglsang et al., 2003). Therefore, the selection of suitable bacteria with optimal proteolytic activity is important for these products to exhibit ACE-inhibitory properties (López-Fandiño et al., 2006). The content of potent ACE-inhibitory peptides depends on the balance between their formation and further breakdown (Gobbetti et al., 2004b). Increasing health-consciousness among consumers has boosted the demand for low-fat yogurt with additional health benefits, leading to an increase in yogurts containing prebiotics and probiotics.

Interest in the role of prebiotics as functional food ingredients has been increasing rapidly over the past few years. A prebiotic is an ingredient that allows the growth and activity of selected gastrointestinal microflora and thereby confers benefits upon the well-being and health of the consumer. Prebiotics are now considered to be one of the practical and efficient means for manipulating the gut microflora, provided the health-promoting species are present in the bowel. The most common prebiotics include inulin and oligofructosaccharides which are found in many vegetables, including onion, asparagus, Jerusalem artichoke and chicory root. Yogurts are among the main dairy products in which prebiotics are commonly added. The properties of inulin as a fat replacer in low-fat/no-fat

dairy products are attributed to its capacity to form microcrystals that interact with each other forming small aggregates, which occlude a great amount of water, creating a fine creamy texture that provides a mouth sensation similar to that of fat (Bot et al., 2004).

All fermented milk products contain live lactic acid bacteria (LAB), unless they are pasteurised after fermentation. The addition of probiotics to these products is a natural way of enhancing the functionality of these products. The most common functional dairy products are therefore, those with probiotic bacteria, quite frequently enriched with prebiotics (Saxelin et al., 2003). Their consumption has long been associated with good health given that fermented products can contain probiotics, prebiotics or both (Stanton et al., 2005). Yogurt in general is considered a good vehicle for probiotics (Brannon, 2006). Mounting scientific evidence indicates that ingestion of certain microbial cultures exerts health benefits not only in the gastrointestinal tract, but also in the respiratory and urogenital tracts. Probiotics are defined as 'live microorganisms that when administered in adequate amounts confer a health benefit on the host' (FAO/WHO, 2002). As many probiotic bacteria are sensitive to stresses such as oxygen, heat and acid exposure, they perform poorly in many food environments, particularly in fermented foods, which can be highly acidic. Products with a short shelf-life such as yogurt and fermented milks are therefore, the most common probiotic foods available (Stanton et al., 2005). Probiotic products containing *Lactobacillus acidophilus*, *Bifidobacterium* spp. and *L. casei* are becoming increasingly popular and yogurt containing these organisms can be considered a functional probiotic product (Shah, 2007).

To realize the health benefits, the probiotics in yogurt must be viable and available at a concentration of at least 10^6 CFU per gram of the product. However, studies have shown that most probiotic foods have a low population of probiotics and that these organisms are not able to survive the storage period of yogurts (Shah, 2007). To improve a probiotic's viability and vitality, as well as its survival chances in the digestive tract and its subsequent attachment and growth, a prebiotic can be included (Brannon, 2006; Vasiljevic and Shah, 2008). The most common prebiotics are inulin and fructo-oligosaccharides, as they resist digestion by gastric acid and pancreatic enzymes *in vivo* (Cummings et al., 2001; Bruno et al., 2002). When processed and combined with water, inulin has a texture and mouthfeel similar to fat. Therefore, inulin is also used as a fat replacer in dairy products such as low-fat yogurt (Brannon, 2006).

In the last few years the consumer interest has turned towards low-fat yogurts. This changing trend has generated interest in solving the major textural problem of low-fat

yogurts, namely, whey separation. Spontaneous syneresis refers to the loss in the ability of the yogurt gel to entrap all the serum phase due to the weakening of the gel network (Lucey, 2002). Moreover, yogurt texture is extremely fragile. As a result, mechanical handling of the product is difficult. Some of the common methods adopted by the manufacturers to address this problem have been to increase the level of non-fat milk solids or to add sugar, proteins, natural or synthetic gums and stabilizers. Another suggested method for improving the texture of low fat yogurt is enzymatic stimulation of protein interactions in milk (Faergemand et al., 1999; Lorenzen, 2002; Shah, 2003; Welman and Maddox, 2003; Ozer et al., 2007; Xu et al., 2008). However, these methods are limited when faced with the increasing consumer demand of low-fat/sugar products with no additives and stabilizers (Jolly et al., 2002). A viable alternative to this is the use of EPS producing cultures (De Vuyst et al., 2003). Hence, the in situ use of generally recognized as safe, food grade, EPS-producing strains of LAB as functional starters in fermented food products appears to be a feasible solution.

Low-fat yogurts are known to have certain textural problems such as whey separation. Exopolysaccharides (EPS) from LAB can help prevent whey separation as well as improve the rheology, texture and ‘mouthfeel’ of such fermented milk products (Jolly et al., 2002; Welman and Maddox, 2003). However, spontaneous appearance of whey on surface of set yogurt, especially low-fat yogurt, remains a major concern to the manufacturers. This also affects consumer acceptability of the product. One approach to solve this problem relies on the in situ use of generally recognized as safe, food grade, exopolysaccharide (EPS)-producing strains of LAB (DeVuyst et al., 2003). Much of the work related to EPS producing starters have concentrated on their influence on the viscosity, and rheology and texture of yogurt (Hassan et al., 2003a; Zisu and Shah, 2003; Lucey, 2004; Doleyres et al., 2005; Guzel-Seydim et al., 2005; Amatayakul et al., 2006a; Folkenberg et al., 2006b; Girard and Schaffer-Lequart, 2007; Purwandari et al., 2007). Some researchers have studied the effect of added inulin on the rheological and sensory properties of yogurt (Dello Stafollo et al., 2004; Guven et al., 2005; Kip et al., 2006). So far no work has been carried out to study the influence of EPS producing starters on the stability of the ACE-inhibitory activity of fermented milks such as yogurt, inulin containing yogurt or probiotic yogurt. Also very little work has been carried out to monitor the changes in the textural and rheological properties of such yogurts made from EPS producing strains of starters during storage. We have carried out preliminary work to examine the influence of varying levels of Raftiline HP (2 and 3%) on the physico-chemical properties of yogurt with specific reference

to viability, proteolysis and ACE-inhibition and textural properties and have found that inclusion of inulin improved ACE-inhibitory and textural properties of low-fat yogurt (Ramchandran and Shah, 2008b, 2009c).

In the present study, we have comprehensively examined the influence of the EPS producing culture against a non-EPS producing culture; i) on the viability of *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus*, changes in pH and lactic acid content, proteolysis and ACE-inhibitory activity, as well as on the firmness, spontaneous whey separation and rheological parameters during storage of the low-fat yogurt at 4 °C. ii) in the presence of a specific level of Raftiline (3%) on the viability of *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus*, changes in pH and lactic acid content, proteolysis and ACE- and α-glucosidase-inhibitory activities, as well as on the firmness, spontaneous whey separation and rheological parameters during storage of low-fat yogurt at 4 °C for 28 d, and iii) on the viability of *S. thermophilus*, *L. delbrueckii* ssp. *bulgaricus*, *L. acidophilus*, *L. casei* and *B. longum*; changes in pH and lactic acid content; proteolysis and ACE-inhibitory activities; as well as on the firmness, spontaneous whey separation and rheological parameters of low-fat inulin-containing probiotic yogurt during refrigerated storage (In line with the current industry trend of using 2-3 probiotic cultures along with the yogurt cultures, in this study the overall impact of the three probiotics included was examined and not of each probiotic separately). The yield of EPS was also monitored during the period of storage.

6.2 Materials and Methods

6.2.1 Propagation of yogurt starters

Two strains of *Streptococcus thermophilus*, one EPS producer (1275) and one non-EPS producer (1342) were obtained from the Starter Culture Collection of Victoria University (Werribee, Vic, Australia). *Streptococcus thermophilus* 1275 has been established as producing capsular as well as ropy EPS (Zisu and Shah, 2003). *Lactobacillus delbrueckii* ssp. *bulgaricus* 1368 was obtained from Australian Starter Culture Research Centre Ltd. (Werribee, Vic, Australia). The probiotic organisms used were *L. casei* 15286 and *L. acidophilus* 4461, acquired from the Starter Culture Collection of Victoria University (Werribee, Vic, Australia) and *B. longum* 5022 obtained from Australian Starter Culture Research Centre Ltd. (Werribee, Vic, Australia). All three organisms were activated as described in Section 3.2.1.

6.2.2 Preparation of low-fat yogurt

Low-fat yogurt was prepared using skimmed milk (Skinny Milk, Parmalat Australia Ltd., Brisbane, QLD, Australia) that was standardized to 12% total solids with skim milk powder. The method of yogurt making is described under Section 4.2.3. Two batches of yogurt were prepared - one using the non-EPS strain of *S. thermophilus* 1342 (NEY; control batch) and the other using the EPS producing strain of *S. thermophilus* 1275 (EY; experimental batch).

Similarly two types of inulin containing yogurt were prepared - one using the non-EPS strain of *S. thermophilus* 1342 (NEPSY) and the other using the EPS producing strain of *S. thermophilus* 1275 (EPSY).

Two batches of probiotic yogurt were also prepared - one using the strain of *S. thermophilus* 1342 that did not produce EPS (NEPY**;** control batch) and the other using the EPS producing strain of *S. thermophilus* 1275 (EPY**;** experimental batch).

6.2.3 Sampling of yogurt mixes and yogurt for analyses

The samples of inoculated yogurt mixes (0 h) were removed prior to incubation for determining the viable counts, pH, lactic acid content and for measuring proteolysis and ACE-inhibition activity. Samples of all the types of low-fat yogurt were removed from the refrigerated storage at 18 h post-manufacture. This was referred to as d 1 sample. Samples were also removed at d 7, 14, 21 and 28 of storage at 4 °C and analysed for changes in pH, lactic acid content, viability of starter cultures, yield of EPS, proteolysis by *o*-phthaldialdehyde (OPA) method and ACE-inhibitory activity, as well as for firmness, spontaneous whey separation and rheological parameters.

6.2.4 Preparation of filtrates for proteolytic and ACE-inhibitory activities

The filtrates of inoculated samples (0 h) and low-fat yogurt samples stored at 4 °C were prepared and stored as described under Section 4.2.6.

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6.2.5 Measurement of pH

The changes in pH in the yogurts during preparation and storage were measured as indicated under Section 4.2.5.

6.2.6 Determination of lactic acid

The concentration of lactic acid (mg/100g) in all samples of low-fat yogurt stored at 4 °C as well as the 0 h samples of mixes were determined by high performance liquid chromatography (HPLC) as described in Section 4.2.10.

6.2.7 Determination of viability of organisms

The growth and viability of the starter cultures, *S. thermophilus* 1275 and *L. delbrueckii* ssp. *bulgaricus* 1368, in the fresh and stored low-fat yogurts was determined by pour plate technique using M17 agar and MRS agar, respectively (Dave and Shah, 1996). The counts were expressed as CFU per gram of yogurt sample.

In probiotic yogurts, the selective enumeration of yogurt starters and probiotics in freshly inoculated mixes (0 h) and in the low-fat yogurts stored at 4 °C was carried out at weekly intervals by pour plate technique. The selective media used were M17 agar for *S. thermophilus*, reinforced clostridial agar for *L. delbrueckii* ssp. *bulgaricus*, MRS-sorbitol agar for *L. acidophilus*, *L. casei* agar for *L. casei* and MRS-NNLP (nalidixic acid, neomycin sulphate, lithium chloride and paromomycin sulphate) agar for *B. longum* (Ravula and Shah, 1998; Tharmaraj and Shah, 2003). The prepared plates were incubated in anaerobic jars (with the exception of *S. thermophilus*, which was incubated aerobically) at their optimum temperatures. The counts were expressed as $\log_{10}$ CFU per gram of yogurt sample. In the results presented, the corresponding 0 h counts were subtracted from those of the control and experimental yogurts so that the actual changes in $\log_{10}$ counts due to growth and survival of the organisms could be determined.

6.2.8 Determination of crude EPS content

The quantity of crude EPS in the EY samples was measured during the storage period of 28 d at 4 °C by the method of van Geel-Schutten et al. (1998) as described by Purwandari et al. (2007) with some modifications. The method involved centrifugation of 50 g of yogurt at 11000 × *g* for 10 min at 4 °C. The supernatant containing the EPS was collected and mixed with 2 volumes of cold ethanol and left at 4 °C for about 18 to 20 h to precipitate the EPS. This was followed by centrifugation at 11000 × *g* for 15 min at 4 °C. The precipitates thus obtained were dissolved in 20 mL of MilliQ water and mixed with 500 µL of 80% trichloroacetic acid and left at 4 °C for another 18 to 20 h to precipitate the proteins. This was followed by centrifugation at 2000 × *g* for 15 min at 4 °C. The protein-free supernatant was collected and mixed with 2 volumes of cold ethanol and left at 4 °C for 18 to 20 h to re-

precipitate the EPS. The EPS precipitates were collected by centrifuging at 2000 × *g* for 15 min at 4 °C. The steps of protein precipitation and EPS re-precipitation were repeated once more and the EPS finally collected by centrifuging at 2000 × *g* at 4 °C for 15 min. The collected crude EPS was dried at 40 °C until two consecutive weights did not show a difference of more than 0.001 g. The results were expressed as milligrams of crude EPS per 100 grams of yogurt.

6.2.9 Extent of proteolysis by OPA method

The extent of proteolysis was determined by measuring the free amino acid content in filtrates of yogurt mixes (0 h) as well as of low-fat yogurt samples as described in Section 3.2.4 The readings of the 0 h samples as well as the reagent blank were deducted from the corresponding readings of fresh and stored yogurt samples to obtain the amount of free amino acids released as a consequence of the proteolytic activity of the starter cultures.

6.2.10 Determination of ACE-inhibitory activity

The ACE-inhibitory activity and protein content was determined in the filtrates of yogurt mixes as well as of the low-fat yogurts as described in Section 3.2.6.

6.2.11 α -Glucosidase-inhibitory activity

The α-glucosidase (α-glu)-inhibitory activity of the filtrates of yogurt mixes as well as of the low-fat yogurts was measured as described in Section 3.2.7.

6.2.12 Firmness of yogurt

The firmness of the low-fat yogurts, measured as the force required to break the gel, was determined using a texture analyzer TA-XT.2 as described in Section 4.2.12.

6.2.13 Spontaneous whey separation

Spontaneous whey separation in the stored low-fat yogurt, indicative of the whey expelled from the gel without the application of external pressure, was determined as described in Section 4.2.11.

6.2.14 Rheological measurements

The low-fat yogurt stored at 4 °C was gently stirred 5 times prior to rheological analysis. The viscoelastic properties were determined by small amplitude oscillatory

measurement (SAOM) (described in Section 4.2.13). A portion of the stirred samples was loaded on the inset plate and presheared at a shear rate of 500 per s for 30 s and then equilibrated for 150 s to allow structure rebuilding before SAOM was performed. Pre-shearing at high shear rate was required to erase the processing history of these semisolid materials. The newly formed structures would clearly be dependent on the medium composition. Moreover, the pre-shear eliminates residual stress or anisotropy, which consequently, gives reproducible results. These restructured gels in general have mechanical spectra resembling those of the set-type yoghurt with viscoelastic moduli being 8-10 times lower (Jaros & Rohm, 2003). The samples were first subjected to a frequency sweep test using a frequency ramp from 0.1 to 10 Hz at a constant strain of 0.5% (determined from an amplitude sweep at 1 Hz) to ascertain the viscoelastic properties. The shear rate, storage modulus, loss modulus and damping factors were recorded for all the samples. This was followed by a shear rate sweep to generate the flow curves. The shear stress was measured as a function of shear rates from 0.1 to 100 per s (upward and downward sweeps). The flow behaviour of the samples was determined by using the Herschel-Bulkley model (described in Section 6.2.11). The hysteresis loop area between the upward and downward curves was also calculated using the RheoWin Pro software (Anton Paar).

6.2.15 Experimental design and statistical analysis

The yogurt making experiment was designed with culture (strains of *S. thermophilus*) and replications as the main plot and time as the sub-plot. This block was replicated thrice with two sub-samplings. The results of the various determinations were analysed as split plot in time using the General Linear Model procedure of SAS system (SAS, 1996). The data of EPS concentration was analysed by one-way Anova and Tukey's test for multicomparison of the means. Correlational analysis was employed, where appropriate, using Microsoft Excel Statpro software. The level of significance was set at $P = 0.05$.

6.3 Results and Discussion

6.3.1 Changes in pH

On an average EY took more time (287 min) than NEY (277 min) to reach the pH of 4.5 ± 0.1. An early gelation in the presence of EPS producing cultures has been reported to inhibit the mobility of the growing organisms (Hassan et al., 2001). This could in part explain the possible reason for the increased fermentation time observed in EY. On the

contrary, Doleyres et al. (2005) have reported that yogurt prepared using EPS-producing cultures fermented faster than those prepared using non-EPS cultures.

During storage at 4 °C, both the types of yogurt showed a sharp decrease ($P < 0.05$) in pH at d 7 followed by a sharp increase ($P < 0.05$) at d 21 of storage (Table 6.1). This was concomitant with the significant increase in lactic acid content of the yogurts (Table 6.1). There were no significant variations in pH of yogurts during the other periods of storage. Purwandari et al. (2007) also observed that *S. thermophilus* 1275 stopped producing acid in yogurt after 7 days of storage at 4 °C. However, the pH at the end of storage was almost the same as that at the start (d 1) for both, NEY and EY. The variation in the strain of *S. thermophilus* (NEY strain vs. EY strain) did not have any influence on the changes in pH throughout the storage period. The increase in pH towards the end of storage period could be attributed to the ability of *S. thermophilus* to produce certain basic metabolites (Tinson et al., 1982). This is contrary to the observations of Ozer et al. (2007), Purwandari et al. (2007) and Al-Kadamany et al. (2003) who observed a continuous decrease in pH of yogurts during storage whereas Salvador and Fiszman (2004) did not observe any change in pH of yogurt during storage. These differences could be due to the different types and combination of starters used for yogurt making by these workers.

The acidification time to reach the pH of 4.5 in the presence of EPS-producing *S. thermophilus* (EPSY) was 273 min, which was less than in the presence of the non-EPS-producing strain (NEPSY, 282 min). A similar observation was made by Doleyres et al. (2005) and Purwandari et al. (2007).

The changes in pH during storage of the low-fat yogurts, NEPSY and EPSY, are given in Table 6.2. There was no difference ($P > 0.05$) in the pH between NEPSY and EPSY throughout the storage period. This is similar to the observation of Doleyres et al. (2005) who concluded that the low amount of EPS does not have any influence on post-acidification activity. During storage at 4 °C, NEPSY showed a significant ($P < 0.05$) decrease in pH at d 7 and 14 while EPSY showed a decrease ($P < 0.05$) only at d 7. Both the yogurts showed a dramatic increase ($P < 0.05$) in pH on d 21 to values similar to those of d 1. Considering that there was no increase in the lactic acid content of both the yogurts after d 7 (Table 6.2), it is possible that some basic metabolites could have been produced at the low temperature (4 °C) that caused the increase in pH at d 21 (Tinson et al., 1982).

Table 6.3 shows the changes in pH of the low-fat probiotic yogurts during storage at 4 °C for 28 d. Both NEPY and EPY showed a significant ($P < 0.05$) decrease in pH at d 7 and an increase ($P < 0.05$) at d 21. A similar observation was made by Ramchandran and

Shah (2009a) in low-fat yogurts made from EPS and non-EPS producing strains of *S. thermophilus*. The changes in pH during storage were also found to be similar in low-fat yogurts containing inulin (Ramchandran and Shah, 2008b). Özer et al. (2005) have also observed a drop in the pH of yogurts containing inulin to 4.27 to 4.42, during the 14 d storage period at 4 °C. The ability of *S. thermophilus* to produce some basic metabolites during the later part of storage could be the possible reason for the increase in pH observed at d 21 and 28 (Tinson et al., 1982). Thus, the combined presence of EPS, inulin and probiotics did not influence ($P > 0.05$) the changes in pH during storage. This is similar to the observation of Doleyres et al. (2005), who concluded that the low amount of EPS does not have any influence on post-acidification activity.

6.3.2 Changes in lactic acid

The concentration of lactic acid (mg/100g) in the control and experimental yogurts (EY and NEY) stored at 4 °C for 28 d is shown in Table 6.1. The amount of lactic acid produced was not influenced by the strain of *S. thermophilus* used for making yogurt. All the yogurts showed an increase in lactic acid content ($P < 0.05$) by 0.37 to 0.39 mg/100g during the first week (d 7) of storage after which there were no significant changes in the concentration of lactic acid. Amatayakul et al. (2006a) did not observe any influence of the type of starter on the amount of lactic acid produced, although they reported slight increases in the lactic acid content during the 28 d storage period. On the contrary, Guzel-Seydim et al. (2005) reported significantly higher concentration of lactic acid in yogurts produced with a ropy cultures and a decrease in the content after 14 d storage at 4 °C. These variations could be due to the differences in the strains of organisms used for yogurt making.

The lactic acid content of NEPSY and EPSY as observed during storage at 4 °C for 28 d is also given in Table 6.2. Both the yogurts exhibited a significant ($P < 0.05$) increase in the lactic acid concentration at d 7 and the values did not change during the remaining storage period. There was no difference ($P > 0.05$) in the lactic acid content of NEPSY and EPSY throughout the storage period, except at d 1 ($P < 0.05$).

The concentration of lactic acid (mg/100g) in the yogurts EPY and NEPY during storage at 4 °C is shown in Table 6.3. Both type of yogurts showed a significant ($P < 0.05$) increase in the lactic acid content at d 7 after which the increases were not significant ($P > 0.05$) for the rest of the storage period. There was no difference in the amount of lactic acid produced in NEPY and EPY. This is similar to the changes in pH observed (Table 6.3) confirming that EPS, inulin and probiotics together did not influence the post-acidification in

yogurts. Ramchandran and Shah (2009a) have made a similar observation earlier in EPS containing yogurts. Some researchers, however, observed an increase in lactic acid content during storage of yogurts at 4 °C (Özer et al., 2005; Amatayakul et al., 2006a).

6.3.3 Viability of organisms in yogurt

The change in the number of EY strain of *S. thermophilus* 1275, NEY strain of *S. thermophilus* 1342 and *L. delbrueckii* ssp. *bulgaricus* 1368 during storage is reported in Table 6.4. Among the strains of *S. thermophilus*, the counts of the NEY strain remained similar throughout the storage period, while the EY strain showed an increase ($P < 0.05$) at d 14 and a decrease ($P < 0.05$) at d 28, although their numbers at d 28 were similar to those at the start of storage (d 1). Also, the increase in the counts of EY strain of *S. thermophilus* was significantly higher than that of NEY strain of *S. thermophilus* at d 14 and 21 of storage indicating some protective effect of EPS. Purwandari et al. (2007) however, observed an increase in counts of EY strain of *S. thermophilus* only during the first week of cold storage of yogurt prepared using only *S. thermophilus*. On the other hand, the counts of *L. delbrueckii* ssp. *bulgaricus* in control (NEY) were lower ($P < 0.05$) than those in experimental (EY) yogurts. They showed sharp decreases ($P < 0.05$) in NEY in the first and second week of storage whereas in EY they decreased ($P < 0.05$) during the first and third week of storage at 4 °C. It was also observed that during fermentation, *L. delbrueckii* ssp. *bulgaricus* exhibited an increase of only one log cycle in NEY while in EY the increase was by two log cycles. This is reflected in the higher numbers ($P < 0.05$) of *L. delbrueckii* ssp. *bulgaricus* in EY as compared to that in NEY. This implies that *L. delbrueckii* ssp. *bulgaricus* was able to grow and survive better in the presence of EPS producing strain of *S. thermophilus*. Thus, EPS appears to have a protective effect on *L. delbrueckii* ssp. *bulgaricus* and to some extent on *S. thermophilus*. Amatayakul et al. (2006a) have also observed the protective effect of EPS on *L. delbrueckii* ssp. *bulgaricus*. Salvador and Fiszman (2004) observed significant decrease in viability of S. *thermophilus* and *L. delbrueckii* ssp. *bulgaricus* in skimmed yogurt after 15 d of storage at 10 °C.

The changes in the viability of the yogurt starters (EPSY and NEPSY) during storage at 4 °C are presented in Table 6.5. There were no changes ($P > 0.05$) in the counts of the non-EPS producing strain of *S. thermophilus* throughout the storage period whereas the EPS-producing strain showed an increase ($P < 0.05$) at d 14 followed by a decrease ($P < 0.05$) at d 28, to numbers similar to those at d 1. Also, there were no differences ($P > 0.05$) between their numbers in NEPSY and EPSY during storage, except at d 21. The counts of *S.*

thermophilus in both the yogurts were marginally higher in the presence of inulin than those observed in an earlier study, in the absence of inulin (Ramchandran and Shah, 2009a). In contrast, there were significant decreases in the counts of *L. delbrueckii* ssp. *bulgaricus* in NEPSY at d 7 and 14 while there were no changes in counts in EPSY all through the storage period. Moreover, the counts were higher ($P < 0.05$) in EPSY as compared to those of NEPSY throughout the storage period. This confirms the protective effect of EPS on the survival of *L. delbrueckii* ssp. *bulgaricus* during storage which was not affected by the presence of inulin. Amatayakul et al. (2006a) also observed the protective effect of ropy EPS on *L. delbrueckii* ssp. *bulgaricus*.

Figure 6.1 exhibits the changes in counts ($\Delta \log_{10}$ CFU/g) of the yogurt starters *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* during refrigerated storage of NEPY and EPY. In general, the counts of EPS producing *S. thermophilus* was lower ($P > 0.05$) than those of non-EPS producing *S. thermophilus*, the difference being significant only at d 1. The counts were similar ($P > 0.05$) in both the types of yogurt throughout the storage period. However, the counts of *L. delbrueckii* ssp. *bulgaricus* in EPY were higher ($P < 0.05$) than those in NEPY from d 7 onwards. The count showed a reduction at d 7 and d 21 in NEPY but remained stable in EPY throughout the storage period. This confirms the protective effect of EPS on the survival of *L. delbrueckii* ssp. *bulgaricus*. Such an effect has been reported by other researchers as well (Amatayakul et al., 2006a).

The changes in the survival of the probiotics are given in Table 6.6. No change ($P > 0.05$) in the delta log counts of *L. casei* and *B. longum* in both NEPY and EPY and of those of *L. acidophilus* in NEPY were observed throughout the storage period. In EPY, a significant ($P < 0.05$) increase in numbers of *L. acidophilus* was observed only at d 14, when the counts were higher ($P < 0.05$) than those of NEPY. Lankaputhra and Shah (1995) have reported that *B. longum* and *L. acidophilus* showed good survival in acidic conditions. In our study, the population of all the probiotics was $\geq 2 \times 10^7$ CFU/g at d 28 in both NEPY and EPY. This confirms that the probiotic cultures remained viable in the product until the end of storage (28 d) which is satisfactory for the yogurts to be claimed as probiotic yogurt. Yogurts generally have a shelf life of 20-40 days and viability of probiotic bacteria in products over long shelf life at refrigeration temperature is generally reported to be poor (Dave and Shah, 1997). Several researchers have reported poor survival of probiotics in yogurt during refrigerated storage (Gilliland and Speck, 1977; Dave and Shah, 1997). However, synergistic growth-promoting effects have also been observed between probiotics and/or yogurt starters (Hansen, 1985; Samona and Robinson, 1994) which could explain the improved stability of

the probiotics during storage of yogurt. The presence of inulin could also have contributed to the survival of the probiotics during storage of the NEPY and EPY. Desai et al. (2004) have reported that the viability of probiotic lactobacilli in fermented milk was better in the presence of inulin. Similarly, Capela et al. (2006) and Özer et al. (2005) have reported improvement in the survival of probiotics in yogurts in the presence of inulin. Moreover, the continued proteolysis (Figure 6.3) could have provided the essential growth factors in the form of peptides and amino acids to improve the survival of the probiotics in the product. However, the combined presence of EPS and inulin did not appear to have any specific protective effect on the survival of probiotics although some improvement in the survival of *L. acidophilus* was observed.

6.3.4 Crude EPS content

The EPS content (mg/100g) during storage of EY is presented in Table 6.4. During fermentation EY strain of *S. thermophilus* produced 37.43 mg of EPS per 100 grams of yogurt. The EPS content decreased ($P < 0.05$) to almost one-third the original content at d 7 of storage. There were no significant changes in the content thereafter. The decrease in EPS content could be attributed to the presence of enzymes capable of degrading EPS (Degeest et al., 2002). Purwandari et al. (2007) have also made a similar observation. However, Amatayakul et al. (2006a) have reported an increase in the EPS content during the 28 d storage while Doleyres et al. (2005) found the content to be stable during the 4 week storage period. Variations in the method of estimating the EPS, differences in the types of EPS as well as strain variations could be the possible reasons for the differences observed.

The yield of crude EPS (mg/100g) in EPSY is shown in Table 6.5. The EPS content was similar ($P > 0.05$) throughout the storage period, except for a sharp (~ 4 times) increase ($P < 0.05$) at d 14 followed by decrease ($P < 0.05$) at d 21. The increase at d 14 could be due to the enhanced numbers ($P < 0.05$) of the EPS producer (*S. thermophilus* 1275) observed at d 14 (Table 6.9) while the decrease in EPS content could be attributed to the presence of enzymes capable of degrading EPS (Degeest et al., 2002). Also, the yield of EPS was higher in the presence of inulin, particularly from d 7, than those reported in our previous work in yogurts without added inulin (Ramchandran and Shah, 2009a). Doleyres et al. (2005) found that the EPS content in yogurt remained stable during the 4 wk storage at 5 °C while Amatayakul et al. (2006a) reported an increase in the concentration of EPS in yogurt made with ropy starter cultures but not in yogurt made using capsular EPS-producing starter cultures. Purwandari et al. (2007) observed a decrease in the EPS content of yogurts during

storage. As yet there are no reports on the changes in EPS content of inulin containing yogurts.

The concentration of crude EPS in EPY at various time periods of storage is given in Table 6.6. No EPS residue was observed in NEPY samples. A steady increase in the concentration of EPS was observed until d 21 (almost 2.4 times), the increase being significant ($P < 0.05$) only at d 21. Thereafter, there was a sharp decrease ($P < 0.05$) in the concentration of EPS at d 28. However, it was interesting to observe that inclusion of probiotics increased the yield of EPS (by about 5 mg/100g) in the yogurts at d 1 than that in yogurts prepared only with the yogurt starters (Ramchandran and Shah, 2009a). The decrease in EPS content of yogurts during storage could be attributed to the presence of enzymes capable of degrading EPS (Degeest et al., 2002). Purwandari et al. (2007) have also made a similar observation. However, Amatayakul et al. (2006a) have reported an increase in the EPS content during the 28 d storage while Doleyres et al. (2005) found the content to be stable during the 4 week storage period. Variations in the method of estimating the EPS, differences in the types of EPS as well as strain variations could be the possible reasons for the differences observed.

6.3.5 Extent of proteolysis

The extent of proteolysis, as measured by the difference in the absorbance (ΔA_{340}) of the yogurt filtrates (NEY and EY) and the absorbance of the corresponding 0 h filtrates, is shown in Table 6.7. Both the yogurts showed similar degree of proteolysis at d 1 and 7. A significant increase was observed at d 14 (0.265 units) for EY but not for NEY. Thereafter, NEY continued to show significant increases but the extent of proteolysis in EY was similar for the last two weeks of storage. The proteolytic activity was similar for EY and NEY during the first two weeks of storage, higher ($P < 0.05$) in EY at d 14 and 21 but towards the end of storage (d 28), NEY showed more ($P < 0.05$) proteolysis. Guzel-Seydim et al. (2005) observed significantly higher proteolysis (measured as tyrosine value) in yogurt prepared using ropy cultures than that prepared using non-ropy cultures, both of which showed an increase at d 14 of storage.

The changes in the extent of proteolysis in NEPSY and EPSY, as measured by the increase in the level of free of amino acids, are shown in Figure 6.2. While the proteolytic activity in NEPSY increased ($P < 0.05$) until d 14, that in EPSY remained stable until d 7 before showing a continuous increase ($P < 0.05$) until d 21. Proteolytic activity was higher ($P < 0.05$) in EPSY than in NEPSY at the beginning (d 1) and then towards the end of the

storage period (d 21 and 28). The increase in extent of proteolysis was much higher in NEPSY (0.24 units) than in EPSY (0.05 units) during the first week of storage, which resulted in a higher ($P < 0.05$) extent of proteolysis in NEPSY at d 7. However, during the third week, the extent of proteolysis increased by 0.156 units in EPSY compared to a negligible decrease in NEPSY, which resulted in increased absorbance values of EPSY towards the end of the storage period. So far no work has been carried out to study the influence of EPS on proteolysis of inulin containing yogurt.

The changes in proteolysis in NEPY and EPY, as measured by the concentration of free amino acids, are depicted in Figure 6.3. The extent of proteolysis was similar in NEPY and EPY until d 14. Thereafter, it was higher ($P < 0.05$) in EPY than in NEPY. Over the storage period of 28 d, both the yogurts showed continued increase in extent of proteolysis being significant ($P < 0.05$) at d 7, 14 and 28 for EPY whereas for NEPY the changes were significant at d 7, 14 and 21. Overall, the increase in level of free amino acids between d1 and d28 was higher for EPY (0.698 units) than for NEPY (0.563 units) indicating that EPS containing yogurts exhibited higher proteolysis in the presence of inulin and probiotics than the corresponding control. Guzel-Seydim et al. (2005) observed significantly higher proteolysis (measured as tyrosine value) in yogurt prepared using ropy cultures than that prepared using non-ropy cultures, both of which showed an increase at d 14 of storage. However, no such study has been conducted with probiotic yogurts.

6.3.6 ACE-inhibition

The changes in percent ACE-inhibitory activity and their corresponding IC_{50} (mg/mL) values of the yogurt filtrates from EY and NEY are depicted in Fig. 6.4. No ACE-inhibitory activity was detected in the 0 h samples of the two types of yogurt mixes indicating that ACE-inhibition observed in the samples were a consequence of the proteolytic activity of the yogurt starters. An increase ($P < 0.05$) in ACE-inhibition was observed at d 7 for both types of yogurt (NEY and EY). Thereafter, while NEY exhibited a decrease ($P < 0.05$) at d 14 followed by an increase ($P < 0.05$) at d 28, EY showed a decrease in ACE-inhibition being significant at d 14 and d 28. Although the ACE-inhibition of EY was higher at d 14 than that of NEY, the IC_{50} values were similar since the soluble protein content (data not shown) was significantly higher in EY compared to that in NEY. The ACE-inhibition was similar in NEY and EY throughout the storage period except at d 14 and d 28 ($P < 0.05$). These changes could be due to the continued proteolysis to varying extents observed in the yogurts (Table 6.7) that resulted in hydrolysis of the existing peptides and

generation of newer peptides having ACE-inhibition potential (Ramchandran and Shah, 2008c). It appears that EPS does not offer any particular protection to the ACE-inhibitory potential of the yogurts. This aspect has not been studied so far.

The ACE-inhibitory activity (%) along with the corresponding IC_{50} (mg/mL) values of the low-fat yogurts EPSY and NEPSY is given in Table 6.8. No ACE-inhibitory activity was observed in the 0 h filtrates of the yogurt mixes. At d 1 and 14, the inhibitory activity was higher ($P < 0.05$) in NEPSY than in EPSY, but at the end of storage it was the reverse. Consequently, the IC_{50} value of EPSY was also lower than that of NEPSY at d 28. The activity was similar ($P > 0.05$) at d 7 and 21. The ACE-inhibitory (%) values were higher in the EPS^+ yogurts in the presence of inulin than those observed in our previous study in EPS^+ yogurts in the absence of inulin (Ramchandran and Shah, 2009a). During storage, the ACE-inhibitory activity increased ($P < 0.05$) in NEPSY at d 7 and dropped ($P < 0.05$) at d 21 before increasing ($P < 0.05$) again at d 28. In case of EPSY, the activity increased ($P < 0.05$) at d 7 but decreased ($P < 0.05$) at d 14 before increasing ($P < 0.05$) at d 28. Although there was no correlation between the extent of proteolysis and ACE-inhibition, the continued proteolytic activity (Figure 6.2) could have resulted in changes in the content of peptides showing ACE-inhibitory potential which caused variations in the activity during storage. Gobbetti et al. (2004b) have also made a similar observation. Additionally, Fuglsang et al. (2003) have reported that a high OPA index does not necessarily indicate higher ACE-inhibition. So far there are no reports on the influence of EPS-producing strains of starters on the ACE-inhibitory potential of yogurt containing inulin.

The percent ACE-inhibitory activity and the corresponding IC_{50} (mg/mL) values of NEPY and EPY are shown in Table 6.9. The ACE-inhibitory activity was similar ($P > 0.05$) in both types of yogurts throughout the storage period. During storage of the yogurts, both NEPY and EPY showed a significant ($P < 0.05$) increase in ACE-inhibition at d 7 followed by a decrease at d 14 and d 21. This was followed by another sharp increase ($P < 0.05$) at d 28. Variations in the ACE-inhibition activity during storage could be attributed to the continued proteolytic activity which could have modified the concentration of the peptides having ACE-inhibition potential. The content of potent ACE-inhibitory peptides appears to rely on a balance between their formation and further breakdown into inactive peptides and amino acids, in turn depending upon storage time and conditions (López-Fandiño et al., 2006). However, there did not appear to be any influence of EPS on the ACE-inhibition activity of the inulin containing probiotic yogurts. The authors have made a similar observation in an earlier study (Ramchandran and Shah, 2009a).

6.3.7 α-Glu-inhibition

The ability of the low-fat yogurts NEPSY and EPSY to inhibit the activity of α-glu with the corresponding IC_{50} values (mg/mL) is shown in Table 6.8. The 0 h filtrates of the yogurts did not show any α-glu-inhibitory activity. Among the two types of yogurts, EPSY showed the inhibitory activity throughout the storage period; the activity increased ($P < 0.05$) at d 7 and then remained stable for the rest of the storage period. On the other hand, the activity was not detected in NEPSY until d 21. In an earlier work, the authors found that *S. thermophilus* 1275 and *L. delbrueckii* ssp. *bulgaricus* 1368 showed α-glu-inhibitory activity in the presence of inulin when grown individually for 6 h at 37 °C (Ramchandran and Shah, 2009b). We also observed that in the absence of inulin, low-fat yogurts with EPS and non-EPS producing strains of *S. thermophilus* did not show any such activity (Ramchandran and Shah, 2009a). It is thus possible that the presence of inulin could have aided the generation of components showing α-glu-inhibitory activity. The higher ($P < 0.05$) α-glu-inhibitory potential of EPSY as compared to that in NEPSY could be due the variation in the strain of *S. thermophilus*. Thus, it appears that in the presence of EPS-producing strain of *S. thermophilus* and inulin, the low-fat yogurts showed some anti-diabetic potential due to their ability to inhibit α-glu.

6.3.8 Firmness of yogurt

The firmness (*g*) of the yogurts EY and NEY measured during storage for 28 d at 4 °C is presented in Figure 6.5. The firmness of yogurt prepared from EPS producing culture (EY) was lower ($P < 0.05$) than that of the control (NEY) at d 1. Both the yogurts showed a significant increase at d 7 after which there was no significant change in their firmness. Throughout the storage period, the firmness of EY was lower ($P < 0.05$) than that of NEY, except at d 14. This clearly indicates that EPS contributes to lowering the firmness of the yogurt gels. Several researchers have made a similar observation using different types of EPS producers (Hess et al., 1997; Hassan et al., 2003b; Amatayakul et al., 2006a; Folkenberg et al., 2006b). The changes in firmness showed a negative correlation ($r = - 0.89$) with the changes in EPS content of EY during storage at 4 °C for 28 d. Folkenberg et al. (2006b) also found an inverse correlation between the presence of EPS and gel firmness. It has been reported that in yogurts made using EPS producing strains, there is incompatibility between EPS and proteins as well as void spaces around EPS producing bacteria that could result in weaker network with lesser protein-protein interactions (Ruas-Madiedo et al., 2002; Hassan et al., 2003b). Also, initiation of gelation at relatively higher pH in these yogurts

could result in the formation of densely aggregated networks causing the EPS to aggregate in the continuous phase as aggregation proceeds with the progress in fermentation (Hassan et al., 2003b). It has also been reported that EPS that are weakly charged and having low molecular weight reduce the firmness of gels (Girard and Schaffer-Lequart, 2007).

Table 6.10 presents the changes in firmness (gf) of the low-fat yogurts EPSY and NEPSY during storage at 4 °C. While the firmness of NEPSY showed a significant ($P < 0.05$) increase at d 7 followed by non-significant increases for the remaining period of storage, that of EPSY remained stable ($P > 0.05$) throughout the storage period. Also, EPSY had lesser firmness ($P < 0.05$) than NEPSY during storage, except at d 1 when both the yogurts had similar ($P > 0.05$) firmness. Interestingly, a comparison to our earlier study (Ramchandran and Shah, 2009a) revealed that the inclusion of inulin did not appear to influence the firmness of EPS^+ yogurt but increased that of EPS^- yogurts. This confirms that fermentation with EPS-producing strains resulted in yogurts with low gel firmness as has been reported by several workers (Amatayakul et al., 2006a; Folkenberg et al., 2006b). Presence of EPS could interfere with the association between casein micelles resulting in less firm coagulum (Ruas-Madiedo et al., 2002). Studies of yogurt microstructure have shown void spaces around EPS producing bacteria that can affect the integrity of the protein matrix (Hassan et al., 1995) which also explains the lower firmness of EPS containing yogurts. The changes in firmness showed a good correlation to the extent of proteolysis observed in NEPSY ($r = 0.95$) and EPSY ($r = 0.86$). Gassem and Frank (1991) reported an increase in the firmness of yogurts prepared from milk proteolysed with microbial proteases.

The firmness (g) of the probiotic yogurts EPY and NEPY is presented in Table 6.11. While there was no significant variation ($P > 0.05$) in the firmness of EPY throughout the storage period, that of NEPY increased significantly ($P < 0.05$) at d 21. Also, there was no difference ($P > 0.05$) in the firmness of NEPY and EPY until d 14 after which the firmness of EPY was lower ($P < 0.05$) than that of NEPY. Earlier studies have reported that yogurts containing EPS-producing cultures have lower firmness (Amatayakul et al., 2006a; Ramchandran and Shah, 2009a). Therefore, the similarity in firmness observed during the first two weeks of storage could be due to the presence of probiotics. The physicochemical properties of EPS have been reported to change when the EPS producers are grown as mixed-strain cultures than when grown individually (Bouzar et al., 1997). This could possibly explain the improved firmness of EPS containing yogurts. So far, no study on the effect of EPS on the firmness of inlulin containing probiotic yogurts has been reported.

6.3.9 Spontaneous whey separation

Figure 6.6 shows the changes in spontaneous whey separation observed during the storage of the two types of yogurt, NEY and EY. Both the types of yogurt showed a significant decrease in whey separation at d 7 after which there were no changes, except in the case of NEY, which showed an increase ($P < 0.05$) to a value similar to that at d 1. However, the extent of whey separation was significantly reduced in EY, clearly indicating the influence of EPS. Similar effect of EPS has been reported by Amatayakul et al. (2006a), Guzel-Seydim et al. (2005) and Hess et al. (1997). The changes in spontaneous whey separation showed a negative correlation with firmness, the correlation being better in EY ($r = - 0.65$) than in NEY ($r = - 0.31$). The better water holding capacity of EPS and the modification in yogurt structure due to the presence of EPS are the plausible explanations for the reduced whey separation. The decrease in whey separation observed during storage could in part be due to protein rearrangement (Ozer et al., 1998) and partly due to reduction in EPS content resulting in a more thermodynamically stable system with better water holding capacity (Hassan et al., 2003b). Also, as EY took a longer time to reach pH 4.5 ± 0.1 than that for NEY, it could have resulted in a more compact structure and hence lower syneresis (Hassan et al., 2003b). Castillo et al. (2006) suggested that faster acidification and coagulation reactions enhanced syneresis in cottage cheese gels. Doleyres et al. (2005) also found that yogurts prepared using EPS producing cultures had better water holding capacity and thereby lower syneresis, and that the water holding capacity increased during storage. Although Folkenberg et al. (2006b) reported a negative correlation between syneresis and firmness in EPS containing yogurts; they observed that syneresis was more pronounced in EPS containing yogurts.

The spontaneous percent whey separation exhibited by NEPSY and EPSY during storage at 4 °C for 28 d is presented in Table 6.10. The appearance of surface whey in NEPSY and EPSY was similar ($P > 0.05$) initially (d 1), but for the rest of the storage period it was lower ($P < 0.05$) in EPSY than in NEPSY. The trend of whey separation in NEPSY during storage showed a decrease until d 14, being significant ($P < 0.05$) only at d 14, followed by significant increases at d 21 and 28; in EPSY also a decrease ($P > 0.05$) was observed until d 14 followed by an increase ($P < 0.05$) to the initial values at d 21 and 28. The EPS content of EPSY showed a good inverse correlation ($r = -0.78$) to the spontaneous whey separation during storage indicating that the yogurts had a better and more stable structure in the presence of EPS that prevented syneresis. However, it was noted that the spontaneous whey separation was relatively higher in EPS^+ yogurts containing inulin in

comparison to those observed in EPS^+ yogurts without inulin (Ramchandran and Shah, 2009a). Amatayakul et al. (2006a) did not find any change in syneresis during storage of yogurt. Several researchers have observed a reduction in syneresis of yogurts made with EPS-producing cultures (Doleyres et al., 2005; Amatayakul et al., 2006a; Ramchandran and Shah, 2009a). The shear-induced microstructure in yogurt made with EPS-producing culture has been shown to consist of compartmentalized protein aggregates between channels containing EPS, which hinders syneresis (Hassan et al., 1995). The improved water holding capacity of yogurts containing EPS, due to the high water-binding property of EPS, is another reason for decreased syneresis (Sodini et al., 2004).

The percent spontaneous whey separation of NEPY and EPY is given in Table 6.11. During the first two weeks of storage, the whey separation was lower ($P < 0.05$) in EPY than in NEPY after which it was similar ($P > 0.05$) in both the yogurts. Despite the increase in the EPS content (Table 6.6) there was no concomitant decrease in the spontaneous whey separation of EPY. This behaviour differs from that observed by the authors in the absence of probiotics in EPS containing low-fat yogurts (Ramchandran and Shah, 2009a). Doleyres et al. (2005) found that yogurts prepared using EPS producing cultures had better water holding capacity and thereby lower syneresis, and that the water holding capacity increased during storage. On the contrary, Folkenberg et al. (2006b) observed that syneresis was more pronounced in EPS containing yogurts. They have suggested that yogurts should have a structure with medium size pores containing EPS to provide a stable structure with minimum syneresis. It appears that inclusion of probiotics might have modified the water holding capacity of EPS which probably affected the water retention capacity of the EPY gels.

6.3.10 Rheological parameters

To model the flow behaviour of the two types of low-fat yogurts, NEY and EY, during storage, the upward flow curves (shear stress) were fitted to the Herschel-Bulkley model to obtain yield stress σ_0, consistency index k and flow behaviour index n (Table 6.12). The yield stress for NEY was higher ($P < 0.05$) than those of EY throughout the storage period suggesting the influence of EPS. This is in agreement with the higher firmness exhibited by NEY than EY (Figure 6.5). Doleyres et al. (2005) and Hassan et al. (2003b) have also made a similar observation. The decreased interactions between protein aggregates due to the presence of EPS in the continuous phase surrounding the aggregate is understood to be responsible for this (Hassan et al., 2003b). However, no change in the yield

stress was observed for EY throughout the storage period, while for NEY the increase was significant only at d 21. The consistency index of EY was, surprisingly, similar to that of NEY until d 14 after which it was lower ($P < 0.05$) than those of NEY. There was no effect of storage time on the consistency index of both NEY and EY. There are conflicting reports for this in literature. Hess et al. (1997) reported a lower consistency index for EPS^+ yogurts, while Doleyres et al. (2005) and Hassan et al. (2003b) have reported a higher consistency index and thus a higher viscosity in EPS-containing yogurt than non-EPS-containing ones. However, variations in the viscosifying effect due to variations in type of cultures producing different types of EPS are known (Sebastiani and Zelger, 1998). No variations in the flow behaviour index (Table 6.12) were observed between NEY and EY nor was there any influence of the time of storage. Doleyres et al. (2005) have also reported that the flow behaviour index of yogurts produced with or without EPS-producing starter culture did not differ significantly. However, the low values (<1) of flow behaviour index confirm the deviation in flow behaviour of yogurts from Newtonian fluids, although there does not appear to be any influence of the presence of EPS.

The thixotropic behaviour of NEY and EY as determined by the hysteresis loop area (Pa/s) between the upward and downward curves (shear rate 0.1 to 100 per s) is presented in Table 6.12. The higher values for NEY indicate slower structural recovery in these samples than in EY. Girard and Schaffer-Lequart (2007) have reported that weakly charged EPS and low-molecular weight EPS allowed best recovery of the texture of milk gels after shearing. A weak gel made up of loosely bound aggregates may rebuild more easily than brittle ones. The values for NEY were higher than those for EY ($P < 0.05$) only at d 14, 21 and 28. This is in concurrence with the higher consistency coefficients of NEY than EY. The correlation of firmness to hysteresis loop area was also better for NEY ($r = 0.75$) than for EY ($r = 0.60$). There was no change in hysteresis area of EY throughout the storage period while an increase ($P < 0.05$) was observed in NEY at d 21 and 28. However, Amatayakul et al. (2006a) found higher loop area values (shear rate 10 to 50 per s) for EPS containing yogurts that varied during storage, while Purwandari et al. (2007) have observed greater hysteresis loop area in EPS containing yogurts at the end of storage. Koksoy and Kilic (2004) have found that an increase in consistency coefficient was associated with increased thixotropy.

The results of frequency sweep of NEY and EY, reported as G' vs log frequency, gave a straight line as shown in Figure 6.7 and 6.8 respectively. The storage modulus (G') of NEY (482 Pa) was higher than that of EY (161 Pa) as was the loss modulus (G'') (131 and 46 Pa respectively). Both G' and G'' increased with storage to 1172 and 305 Pa for NEY and

483 and 128 Pa for EY, respectively. This indicates that NEY had more solid-like (elastic) properties than EY thereby implying that EPS conferred more viscous properties to the yogurt. This is confirmed by the higher ($P < 0.05$) firmness (Figure 6.5) and yield stress (Table 6.12) values of NEY as compared to EY. Extensive particle rearrangement during structure formation resulting in dense clusters of aggregates along with lesser protein-protein interactions could have caused lower G′ values in EY. Purwandari et al. (2007), Doleyres et al. (2005), and Hassan et al. (2003b) have also made similar observations.

The storage and loss moduli (Pa) and damping factor (at 1.5 Hz) of NEPSY and EPSY as obtained from their frequency sweeps are given in Table 6.13. In general, the loss modulus (G″) was lower than the storage modulus (G′) for both the yogurts throughout the storage period indicating that the yogurts exhibited characteristics typical of a weak viscoelastic gel. The values of both G″ and G′ of EPSY were lower ($P < 0.05$) than those of NEPSY during storage. The lower Gvalues indicate that EPS containing yogurt gels had solid-like character, as was also observed by Purwandari et al. (2007). However, the damping factor (tan δ) was similar ($P > 0.05$) for both the types of yogurts throughout the storage period, except at d 7 and 21, when EPSY showed a higher ($P < 0.05$) value than NEPSY. During storage, the tan δ values of both the yogurts decreased with time, being significant ($P < 0.05$) at d 7 and d 14 for EPSY while for NEPSY it was at d 7 only. Decreases in tan δ values indicate that rearrangement of yogurt structures occur during storage to a more solid-like gel (Doleyres et al., 2005). This is consistent with the increase in G′ as shown in Table 6.13. Lucey et al. (1998a) suggested that extensive particle rearrangement during structure formation results in dense clusters of aggregates and lower G′ values. Similar observations regarding G″, G′ and tan δ have been made by other researchers (Hess et al., 1997; Doleyres et al., 2005; Purwandari et al., 2007). The viscosity of the stored yogurt samples decreased with increasing shear rate (Figure 6.9 and 6.10) confirming their non-Newtonian behaviour. The viscosity of EPSY was in general, lower than that of NEPSY. Studies on the effect of EPS on viscosity of yogurt have shown controversial results including an improvement in viscosity (De Vuyst et al., 2003; Guzel-Seydim et al., 2005), a decrease in viscosity (Hess et al., 1997) and no correlation between EPS production and viscosity (Shihata and Shah, 2002). Differences in viscosity of various strains may be due to differences in the intrinsic viscosity of the EPS produced (Ruas Madiedo et al., 2002) or differences in EPS localization within the gel (Sodini et al., 2004).

The upward flow curves (shear stress) of the two types of low-fat yogurts were fitted to Herschel-Bulkley model to study the changes in their flow behaviour during storage. The

values of yield stress (σ_0), consistency index (k) and flow behaviour index (n) thus obtained are presented in Table 6.14. The increase in the values of yield stress (Pa) during storage, for both NEPSY and EPSY was not significant, except on d 28 ($P < 0.05$) for NEPSY. Also, the yield stress of EPSY was lower ($P < 0.05$) than that of NEPSY during storage, except at d 1 when the values were similar ($P > 0.05$). This is in concurrence with the firmness of the yogurts (Table 6.10) as indicated by the strong correlation obtained between the firmness and yield stress values of NEPSY ($r = 0.83$) and EPSY ($r = 0.86$). Polysaccharides, by non-specific entanglements, are believed to prevent the interactions of dispersed particles, which explained the lower yield stress and firmness of EPSY. Hassan et al. (1995) have reported that EPS^+ samples broke down more easily than EPS^- samples because of the presence of fewer protein-protein interactions at the critical sites in the network to overcome. Doleyres et al. (2005) also obtained lowest yield stress values for 13% (total solids) milks fermented with EPS-producing yogurt culture. However, we observed an increase in the yield stress values of EPS+ yogurts in the presence of inulin as compared to those reported in the absence of inulin (Ramchandran and Shah, 2009a). Thus, it appears that presence of inulin molecules aided protein-protein interactions. The consistency index (Pa·s) of EPSY did not change during the storage period, but that of NEPSY increased significantly ($P < 0.05$) at d 28. The consistency index of EPSY was also lower than that of NEPSY throughout the storage period, being significant ($P < 0.05$) only at d 14 and 28. This is in concurrence with the lower viscosity of EPSY than NEPSY as shown in Figure 6.9 and 6.10. This, however, is contrary to the observation of higher consistency coefficients in EPS containing yogurts made by several authors (Doleyres et al., 2005). This may be due to strain variations. Sebastiani and Zelger (1998) have reported that EPS from different EPS-producing strains have different viscosifying effects in yogurts. The flow behaviour index of all the yogurt samples was <1, further confirming their non-Newtonian behaviour. While EPSY maintained its flow behaviour index throughout the storage period, NEPSY showed a significant increase at d 7 and remained stable ($P > 0.05$) thereafter. This increase in flow behaviour index is indicative of an increase in the pseudoplasticity of the NEPSY during storage.

The hysteresis loop area (Pa/s) between the upward and downward curves (shear rate 0.1 to 100 per s), showing the thixotropic behaviour of NEPSY and EPSY (Table 6.14), indicated that EPSY did not show any change in the values throughout the storage period while NEPSY showed continuous increase in the values, being significant at d 14 and 28.

The hysteresis loop area of EPSY was lower than that of NEPSY, being significant ($P < 0.05$) from d 14 onwards. This is contrary to published reports that indicate a high degree of hysteresis in EPS-containing yogurts (Amatayakul et al., 2006a; Folkenberg et al., 2006b). This could be due to differences in the types of EPS produced, which in turn is strain dependent. De Vuyst et al. (2003) have reported that EPS having lower molecular mass showed less-pronounced thixotropic character than EPS having high molecular mass. Hassan et al. (1995) have found that incompatibility between protein particles and EPS produced by bacteria during fermentation could lead to formation of denser and larger aggregates. Koksoy and Kilic (2004) have found that thixotropy in ayran increased with reduction in particle size and higher surface charges. The changes in hysteresis loop area correlated positively with the consistency index of NEPSY ($r = 0.92$) but showed an inverse correlation in EPSY ($r = -0.81$). Variations in rheological and textural properties may arise from differences in the produced EPS (e.g. molecular size or degree of branching) or in the way EPS is incorporated in the protein network in the yogurt gels (Doleyres et al., 2005). The continuously changing balance of repulsive/attractive interactions between EPS and milk proteins over the fermentation process is believed to be an important issue for the final texture and stability of fermented milk (Girard and Schaffer-Lequart, 2008).

The results of frequency sweep of NEPY and EPY, reported as storage modulus (G') vs log frequency, gave a straight line as shown in Figure 6.11 and 6.12, respectively. The storage modulus, at a frequency of 1.5 Hz, of NEY (332.83 Pa) was similar to that of EY (330.67 Pa) so was also the loss modulus (G'') (91.70 and 91.23 Pa, respectively). Both G' and G'' increased with storage to 1128 and 291 Pa for NEPY and 578 and 148 Pa for EPY, respectively. A substantial difference in the values of G' and G'' of NEPY and EPY was observed after d 7. This indicates that after a week of storage NEPY had more solid-like (elastic) properties than EPY. This is confirmed by the higher ($P < 0.05$) firmness (Table 6.11) values of NEPY as compared to EY, observed towards the end of storage. This observation differs from that observed in EPS containing yogurts without any probiotics (Ramchandran and Shah, 2009a).

The shear stress of EPY (on increasing shear rate, corresponding to the upward curve) was higher than that for NEPY at d 1 indicating that EPY has higher apparent viscosity than NEPY. However, this value was lower in EPY than in NEPY for the remaining period of storage indicating a possible decrease in viscosity with storage. The upward flow curves (shear stress) were also fitted to the Herschel-Bulkley model to study the

flow behaviour of NEPY and EPY during storage. The resulting values of yield stress σ_0, consistency index k and flow behaviour index n are presented in Table 6.15. The yield stress for NEPY and EPY was similar at d 1 after which it was higher ($P < 0.05$) for NEPY than for EPY for the rest of the storage period. This implies that the influence of EPS is observable only after a week of storage. This is in agreement with the trend of firmness observed (Table 6.11**)**. No change in the yield stress was observed for EPY throughout the storage period, while for NEPY the increases were significant until d 21. The consistency index of EPY was, surprisingly, similar to that of NEY until d 21 after which it was lower ($P < 0.05$) than that of NEPY. There was no effect of storage time on the consistency index of both NEPY and EPY. No variations in the flow behaviour index (Table 6.15) were observed between NEPY and EPY nor was there any influence of the time of storage. Doleyres et al. (2005) have also reported that the flow behaviour index of yogurts produced with or without EPS-producing starter culture did not differ significantly. However, the low values (<1) of flow behaviour index confirm the deviation in flow behaviour of yogurts from Newtonian fluids, although there does not appear to be any influence of the presence of EPS. Variations in type of cultures producing different types of EPS are known to have varied effects on rheological parameters (Hess et al., 1997; Doleyres et al., 2005).

The thixotropic behaviour of NEPY and EPY as determined by the hysteresis loop area (Pa/s) between the upward and downward curves (shear rate 0.1 to 100 per s) is also presented in Table 6.15. The values for NEPY were similar to those of EPY, except at d 7 and 28, where the values were higher ($P < 0.05$) than those of EPY. This indicates that both yogurts have a similar structure showing similar rate of structural recovery. This is in concurrence with the higher consistency coefficients of NEPY at d 28 than that of EPY. Koksoy and Kilic (2004) have found that an increase in consistency coefficient was associated with increased thixotropy. A good correlation was observed between firmness and hysteresis loop area for NEPY ($r = 0.96$) and for EPY ($r = 0.92$). There was no change ($P > 0.05$) in hysteresis loop area of EPY throughout the storage period while an increase ($P < 0.05$) was observed in NEPY at d 21 and 28. However, Amatayakul et al. (2006a) found higher loop area values (shear rate 10 to 50 per s) for EPS containing yogurts that varied during storage, while Purwandari et al. (2007) have observed greater hysteresis loop area in EPS containing yogurts at the end of storage. The variations in rheological parameters observed could be due to the presence of probiotics along with EPS producing strain of *S. thermophilus*. It has been reported that growing EPS producing strains with non-EPS

producing strains as a mixed strain culture modified the sugar composition as well as the textural characteristics of the EPS produced from that obtained with single pure cultures (Bouzar et al., 1997). So far no detailed rheological study has been conducted on inulin containing probiotic yogurts prepared with EPS producing cultures.

6.4 Conclusion

During storage of the low-fat yogurts EY and NEY, the presence of EPS did not have any influence on changes in pH and lactic acid content although there was a protective effect on *L. delbrueckii* ssp. *bulgaricus* and to some extent on *S. thermophilus*. The EPS content of EY dropped significantly during the first week of storage and remained stable thereafter. There was a significant increase in the extent of proteolysis in EY at d 14 and in NEY at d 14 and 21 of storage. EPS did not appear to have any influence on the ACE-inhibition activity. However, there was a definite influence of EPS in reducing the firmness, spontaneous whey separation and yield stress of low-fat yogurt whereas there was no significant influence on the consistency index and flow behaviour index of the yogurts. Yogurts made from EPS producing starter showed a faster structural recovery after shear and exhibited better viscous properties. Thus, use of EPS producers in low-fat yogurt improved the textural properties of the yogurts without influencing their ACE-inhibition potential.

The combined presence of EPS producing strain of *S. thermophilus* and inulin did not affect pH and lactic acid concentration of the low-fat yogurts (EPSY) but exhibited a protective effect on the survival of *L. delbrueckii* ssp. *bulgaricus* during storage at 4 °C. No change in the EPS content was observed during storage of the inulin containing low-fat yogurts, except for a sharp increase at d 14. The proteolytic activity of yogurt produced with EPS-producing strain of *S. thermophilus* and inulin was higher than the control (NEPSY) at d 1, 21 and 28, indicating a time-dependant effect of EPS. The ACE-inhibitory activity varied with the time of storage in both the types of yogurt, being higher in EPS containing yogurt at d 28. The EPS and inulin containing yogurt showed better α-glu-inhibitory activity as compared to the control. Yogurts made with EPS producing strain of *S. thermophilus* and inulin appeared to have a stable and compact structure as indicated by the reduction in appearance of spontaneous whey separation, lower firmness, G values and yield stress. The consistency index and hysteresis loop area were also lower in the EPS and inulin containing yogurt.

The presence of EPS producing strain of *S. thermophilus* did not affect pH and lactic acid concentration of the low-fat inulin containing probiotic yogurts (EPY). However, EPS exhibited a protective effect on the survival of *L. delbrueckii* ssp. *bulgaricus* during storage at 4 °C but not on that of *L. casei* and *B. longum*. However, some improvement in the survival of *L. acidophilus* was observed in the presence of EPS. The EPS content increased until d 21 after which a significant decrease was observed. Overall, the EPS containing yogurts exhibited higher proteolysis in the presence of inulin and probiotics. No specific protective effect of EPS was observed regarding the ACE-inhibitory activity. The firmness was similar in NEPY and EPY during the first two weeks of storage and no decrease in the spontaneous whey separation of EPY was observed as compared to NEPY thereby indicating that the textural characteristics of EPY differed in the presence of probiotics. The storage and loss moduli, yield stress, consistency index and thixotropic behaviour of both types of yogurt were similar at d 1 and differences in the rheological parameters as a consequence of the presence of EPS were observable only after d 7. Hence, it appears that the influence of EPS on the rheological parameters of yogurt differs in the presence of probiotic organisms. This aspect needs further investigation.

Table 6.1 Changes in pH and lactic acid concentration during storage of control (NEY) and experimental (EY) low-fat yogurts at 4 °C for 28 d.

Type of yogurt	Period of storage (d)				
	1	7	14	21	28
			pH		
NEY	4.53^{aA}	4.40^{bA}	4.34^{bA}	4.58^{aA}	4.53^{aA}
EY	4.52^{aA}	4.44^{bA}	4.33^{bcA}	4.54^{aA}	4.48^{abaA}
SEM			0.024		
			Lactic acid (mg/100g)		
NEY	0.75^{aA}	1.14^{bA}	1.21^{bA}	1.21^{bA}	1.20^{bA}
EY	0.75^{aA}	1.12^{bA}	1.21^{bA}	1.26^{bA}	1.21^{bA}
SEM			0.066		

Values are the means of six observations
SEM = standard error of means
NEY = control yogurt prepared from skim milk standardized to 12% total solids and non-EPS producing strain of *S. thermophilus* 1342; EY = yogurt prepared from skim milk standardized to 12% total solids and EPS producing strain of *S. thermophilus* 1275
[ab]Means in the same row with different alphabets are significantly different for each type of yogurt
[AB]Means in the same column with different alphabets are significantly different for a particular day of storage for each parameter

Table 6.2 Changes in pH and lactic acid content (mg/100g) during storage for 28 d of control (NEPSY) and experimental (EPSY) low-fat yogurts at 4 °C.

Type of Yogurt	Period of Storage (Day)				
	1	7	14	21	28
pH					
NEPSY	4.50^{aA}	4.40^{bA}	4.33^{bcA}	4.50^{aA}	4.49^{aA}
EPSY	4.51^{aA}	4.43^{bA}	4.36^{bA}	4.51^{bcA}	4.50^{acA}
SEM			0.03		
Lactic acid content					
NEPSY	0.97^{aA}	1.14^{bA}	1.17^{bA}	1.20^{bA}	1.20^{bA}
EPSY	0.76^{aB}	1.12^{bA}	1.20^{bA}	1.21^{bA}	1.22^{bA}
SEM			0.05		

Values are the means of six observations
SEM = standard error of means
NEPSY = control yogurt prepared from skim milk standardized to 12% total solids containing 3% inulin and non-EPS producing strain of *S. thermophilus* 1342; EPSY = yogurt prepared from skim milk standardized to 12% total solids containing 3% inulin and EPS producing strain of *S. thermophilus* 1275
[ab]Means in the same row with different alphabets are significantly different for each type of yogurt
[AB]Means in the same column with different alphabets are significantly different for a particular day of storage for each parameter

Table 6.3 Changes in pH and lactic acid concentration during storage of control (NEPY) and experimental (EPY) low-fat probiotic yogurts at 4 °C for 28 d.

Type of Yogurt	Period of Storage (Day)				
	1	7	14	21	28
			pH		
NEPY	4.50^{aA}	4.38^{bA}	4.33^{bA}	4.52^{aA}	4.54^{aA}
EPY	4.49^{aA}	4.42^{bA}	4.36^{bA}	4.53^{aA}	4.53^{aA}
SEM			0.02		
			Lactic acid (mg/100g)		
NEPY	0.74^{aA}	1.10^{bA}	1.20^{bA}	1.20^{bA}	1.26^{bcdA}
EPY	0.86^{aA}	1.06^{bA}	1.18^{bdA}	1.20^{bdA}	1.23^{bcdA}
SEM			0.06		

NEPY = control yogurt prepared from skim milk standardized to 12% total solids containing 3% Orafti HP, probiotics and non-EPS producing strain of *S. thermophilus* 1342; EPY = experimental yogurt prepared from skim milk standardized to 12% total solids containing 3% Orafti HP, probiotics and EPS producing strain of *S. thermophilus* 1275

^{abcd}Means in the same row with different alphabets are significantly different ($P < 0.05$) for each type of yogurt

^{AB}Means in the same column with different alphabets are significantly different ($P < 0.05$) for a particular day of storage for each parameter

Table 6.4 Changes in survival of yogurt starters and EPS content during storage of control (NEY) and experimental (EY) low-fat yogurts at 4 °C for 28 d.

Type of yogurt	Period of storage (d)				
	1	7	14	21	28
Streptococcus thermophilus ($\Delta \log_{10}$ cfu/g)					
NEY	2.06^{aA}	2.06^{aA}	2.17^{aA}	2.12^{aA}	2.03^{aA}
EY	2.12^{aA}	2.06^{aA}	2.40^{bB}	2.31^{bB}	1.99^{aA}
SEM			0.054		
Lactobacillus delbrueckii ssp. *bulgaricus* ($\Delta \log_{10}$ cfu/g)					
NEY	1.52^{aA}	1.00^{bA}	0.33^{bcA}	0.38^{bcA}	0.24^{bcdA}
EY	1.93^{aB}	1.80^{bB}	1.93^{abB}	1.78^{bcB}	1.7^{1bcB}
SEM			0.048		
EPS content (mg/100g)					
EY	37.43 ± 17.75^{a}	11.49 ± 2.68^{b}	11.21 ± 4.05^{b}	14.02 ± 1.43^{b}	16.31 ± 4.19^{ab}

Values are the means of six observations
SEM = standard error of means
NEY = control yogurt prepared from skim milk standardized to 12% total solids and non-EPS producing strain of *S. thermophilus* 1342; EY = yogurt prepared from skim milk standardized to 12% total solids and EPS producing strain of *S. thermophilus* 1275
^{abc}Means in the same row with different alphabets are significantly different for each type of yogurt
^{AB}Means in the same column with different alphabets are significantly different for a particular day of storage for each parameter

Table 6.5 Changes in viability of yogurt starters (Δ $\log_{10}$ CFU/g) and EPS content (mg/100g) during storage for 28 d of control (NEPSY) and experimental (EPSY) low-fat yogurts at 4 °C.

Type of Yogurt	Period of Storage (Day)				
	1	7	14	21	28
Streptococcus thermophilus					
NEPSY	2.06^{aA}	2.09^{aA}	2.19^{baA}	2.15^{aA}	2.11^{aA}
EPSY	2.15^{aA}	2.10^{aA}	2.24^{abA}	2.36^{bB}	2.03^{aA}
SEM	0.05				
Lactobacillus delbrueckii ssp. *bulgaricus*					
NEPSY	1.57^{aA}	0.94^{bA}	0.27^{bcA}	0.22^{bcA}	0.15^{bcA}
EPSY	2.00^{aB}	1.95^{acB}	1.94^{acdB}	1.87^{bcdB}	1.82^{bdB}
SEM	0.04				
EPS content					
EPSY	38.15 ± 26.37^{a}	27.23 ± 12.23^{a}	110.77 ± 9.97^{b}	57.84 ± 18.45^{a}	43.79 ± 24.78^{a}

Values are the means of six observations; Values of EPS are Mean ± SD
SEM = standard error of means
NEPSY = control yogurt prepared from skim milk standardized to 12% total solids containing 3% inulin and non-EPS producing strain of *S. thermophilus* 1342; EPSY = yogurt prepared from skim milk standardized to 12% total solids containing 3% inulin and EPS producing strain of *S. thermophilus* 1275
^{abc}Means in the same row with different alphabets are significantly different for each type of yogurt
^{AB}Means in the same column with different alphabets are significantly different for a particular day of storage for each parameter

Table 6.6 Changes in viability of probiotics and EPS content during storage of control (NEPY) and experimental (EPY) low-fat probiotic yogurts at 4 °C for 28 d.

Type of Yogurt	Period of Storage (Day)				
	1	7	14	21	28
	L. acidophilus 4461 ($\Delta \log_{10}$ CFU/g)				
NEPY	0.56^{aA}	0.59^{aA}	0.56^{aA}	0.59^{aA}	0.59^{aA}
EPY	0.57^{aA}	0.56^{aA}	0.67^{bcB}	0.60^{acA}	0.60^{acA}
SEM	0.04				
	B. longum 5022 ($\Delta \log_{10}$ CFU/g)				
NEPY	0.60^{aA}	0.61^{aA}	0.59^{aA}	0.56^{aA}	0.49^{aA}
EPY	0.63^{aA}	0.67^{aA}	0.62^{aA}	0.56^{aA}	0.58^{aA}
SEM	0.06				
	L. casei 15286 ($\Delta \log_{10}$ CFU/g)				
NEPY	0.64^{aA}	0.63^{aA}	0.59^{aA}	0.64^{aA}	0.66^{aA}
EPY	0.61^{aA}	0.59^{aA}	0.61^{aA}	0.57^{aA}	0.58^{aA}
SEM	0.03				
	EPS content (mg/100g)				
EPY	42.16 ± 14.52^{a}	88.97 ± 24.45^{a}	95.12 ± 30.63^{a}	102.85 ± 14.19^{b}	70.64 ± 5.65^{a}

NEPY = control yogurt prepared from skim milk standardized to 12% total solids containing 3% Orafti HP, probiotics and non-EPS producing strain of *S. thermophilus* 1342; EPY = Experimental yogurt prepared from skim milk standardized to 12% total solids containing 3% Orafti HP, probiotics and EPS producing strain of *S. thermophilus* 1275

^{abc}Means in the same row with different alphabets are significantly different ($P < 0.05$) for each type of yogurt

^{AB}Means in the same column with different alphabets are significantly different ($P < 0.05$) for a particular day of storage for each parameter

Table 6.7 Changes in extent of proteolysis during storage of control (NEY) and experimental (EY) low-fat yogurts at 4 °C for 28 d.

Type of Yogurt	Period of storage (d)				
	1	7	14	21	28
	ΔA_{340}				
NEY	0.536^{aA}	0.536^{aA}	0.502^{aA}	0.685^{abA}	0.879^{bcA}
EY	0.521^{aA}	0.556^{aA}	0.821^{bcB}	0.794^{bcB}	0.722^{bcB}
SEM			0.055		

Values are the means of six observations
SEM = standard error of means
NEY = control yogurt prepared from skim milk standardized to 12% total solids and non-EPS producing strain of *S. thermophilus* 1342; EY = yogurt prepared from skim milk standardized to 12% total solids and EPS producing strain of *S. thermophilus* 1275
[abc]Means in the same row with different alphabets are significantly different for each type of yogurt
[AB]Means in the same column with different alphabets are significantly different for a particular day of storage for each parameter

Table 6.8 Changes in ACE- and α-glucosidase-inhibition (%) and corresponding IC_{50} (mg/mL) in low-fat yogurts stored for 28 d at 4 °C.

	Storage Period									
	D 1		D 7		D 14		D 21		D 28	
	ACE-I	IC_{50}	ACE-I	IC_{50}	ACE-I	IC_{50}	ACE-I	IC_{50}	ACE-I	IC_{50}
NEPSY	22.20^{aA}	2.36	38.28^{bA}	1.15	36.35^{bA}	1.32	12.59^{bcA}	4.20	25.66^{aA}	1.51
EPSY	15.82^{aB}	3.66	39.50^{bA}	1.09	23.78^{bcB}	1.98	17.81^{acA}	2.84	36.30^{bB}	1.28
SEM	2.27									
	α-Glu-I	IC_{50}	α-Glu-I	IC_{50}	α-Glu-I	IC_{50}	α-Glu-I	IC_{50}	α-Glu-I	IC_{50}
NEPSY	ND	-	ND	-	ND	-	6.71^{aA}	0.394	10.79^{aA}	0.180
EPSY	7.63^{a}	0.379	15.68^{b}	0.138	11.16^{ab}	0.211	11.58^{abB}	0.218	14.93^{bB}	0.155
SEM	1.52									

Values are the statistical means of six observations, SEM = standard error of means
NEPSY = control yogurt prepared from skim milk standardized to 12% total solids containing 3% inulin and non-EPS producing strain of *S. thermophilus* 1342; EPSY = yogurt prepared from skim milk standardized to 12% total solids containing 3% inulin and EPS producing strain of *S. thermophilus* 1275
ND = Activity not detected
^{abc}Means in the same row with different alphabets are significantly different within a particular treatment
^{AB}Means in the same column with different alphabets are significantly different for a particular day of storage

Table 6.9 Changes in ACE-inhibition (%) and corresponding IC_{50} (mg/mL) in control (NEPY) and experimental (EPY) low-fat probiotic yogurts stored at 4 °C for 28 d.

Type of Yogurt	Period of Storage (Day)				
	1	7	14	21	28
NEPY					
ACE-I	15.04^{aA}	37.86^{bA}	29.59^{bcA}	23.42^{bcdA}	36.45^{bA}
IC_{50}	3.18	1.13	1.38	2.16	1.51
EPY					
ACE-I	14.82^{aA}	34.91^{bA}	27.60^{bcA}	16.97^{aB}	35.19^{bA}
IC_{50}	3.05	1.29	1.48	2.94	1.27
SEM			1.82		

NEPY = control yogurt prepared from skim milk standardized to 12% total solids containing 3% Orafti HP, probiotics and non-EPS producing strain of *S. thermophilus* 1342; EPY = experimental yogurt prepared from skim milk standardized to 12% total solids containing 3% Orafti HP, probiotics and EPS producing strain of *S. thermophilus* 1275

[abcd]Means in the same row with different alphabets are significantly different ($P < 0.05$) for each type of yogurt

[AB]Means in the same column with different alphabets are significantly different ($P < 0.05$) for a particular day of storage

Table 6.10 Changes in firmness (gf) and spontaneous whey separation (%) during storage for 28 d of control (NEPSY) and experimental (EPSY) low-fat yogurts at 4 °C.

Type of Yogurt	Period of Storage (Day)				
	1	7	14	21	28
Firmness					
NEPSY	74.60^{aA}	84.01^{bA}	85.72^{bA}	89.43^{bA}	90.10^{bA}
EPSY	68.94^{aA}	70.41^{aB}	70.17^{aB}	74.04^{aB}	72.50^{aB}
SEM	2.96				
Spontaneous whey separation					
NEPSY	3.26^{aA}	2.96^{aA}	2.07^{bA}	3.28^{acA}	4.08^{bcA}
EPSY	2.55^{aA}	1.96^{acB}	1.22^{bcB}	2.40^{acdB}	2.56^{acdB}
SEM	0.27				

Values are the means of six observations
SEM = standard error of means
NEPSY = control yogurt prepared from skim milk standardized to 12% total solids containing 3% inulin and non-EPS producing strain of *S. thermophilus* 1342; EPSY = yogurt prepared from skim milk standardized to 12% total solids containing 3% inulin and EPS producing strain of *S. thermophilus* 1275
^{abcd}Means in the same row with different alphabets are significantly different for each type of yogurt
^{AB}Means in the same column with different alphabets are significantly different for a particular day of storage for each parameter

Table 6.11 Changes in firmness and spontaneous whey separation during storage of control (NEPY) and experimental (EPY) low-fat probiotic yogurts at 4 °C for 28 d

Type of Yogurt	Period of Storage (Day)				
	1	7	14	21	28
	Firmness (g)				
NEPY	75.40^{aA}	79.78^{aA}	82.78^{baA}	88.79^{bA}	92.67^{bA}
EPY	75.19^{aA}	72.53^{aA}	80.39^{bacA}	83.51^{bceB}	86.06^{bdeB}
SEM	1.63				
	Spontaneous Whey Separation (%)				
NEPY	2.42^{aA}	2.36^{aA}	1.93^{aA}	2.10^{aA}	2.49^{aA}
EPY	1.43^{aB}	1.64^{aB}	1.51^{aA}	1.94^{acA}	2.32^{bcA}
SEM	0.25				

NEPY = control yogurt prepared from skim milk standardized to 12% total solids containing 3% Orafti HP, probiotics and non-EPS producing strain of *S. thermophilus* 1342; EPY = experimental yogurt prepared from skim milk standardized to 12% total solids containing 3% Orafti HP, probiotics and EPS producing strain of *S. thermophilus* 1275

^{abcde}Means in the same row with different alphabets are significantly different ($P < 0.05$) for each type of yogurt

^{AB}Means in the same column with different alphabets are significantly different ($P < 0.05$) for a particular day of storage for each parameter

Table 6.12 Flow behaviour (predicted by the Herschel-Bulkley model) and hysteresis loop area of control (NEY) and experimental (EY) low-fat yogurts during storage at 4 °C for 28 d.

Storage period (d)	Rheological parameters									
	σ_0 (Pa)		*k* (Pa.s)		n		R^2		Hysteresis (Pa/s)	
	NEY	EY	NEY	EY	NEY	EY	NEY	EY	NEY	EY
1	8.31^{aA}	3.80^{aA}	2.05^{aA}	1.17^{aA}	0.65^{aA}	0.70^{aA}	0.99	0.98	306.28^{aA}	135.85^{aA}
7	9.61^{aA}	5.56^{aA}	1.37^{aA}	1.31^{aA}	0.73^{aA}	0.70^{aA}	0.99	0.98	249.86^{aA}	168.67^{aA}
14	12.75^{aA}	5.17^{aB}	2.35^{aA}	1.38^{aA}	0.63^{aA}	0.70^{aA}	0.98	0.99	385.01^{aA}	165.27^{aB}
21	21.95^{bA}	7.69^{aB}	2.32^{aA}	1.12^{aB}	0.74^{aA}	0.77^{aA}	0.99	0.99	605.03^{bA}	213.70^{aB}
28	23.99^{bA}	11.23^{baB}	2.92^{aA}	1.26^{aB}	0.73^{aA}	0.81^{aA}	0.98	0.98	673.41^{bA}	325.10^{baB}
SEM	2.02		0.43		0.05				63.46	

Values are the means of six observations; SEM = standard error of means
NEY = control yogurt prepared from skim milk standardized to 12% total solids and non-EPS producing strain of *S. thermophilus* 1342; EY = Yogurt prepared from skim milk standardized to 12% total solids and EPS producing strain of *S. thermophilus* 1275
[ab]Means in the same column with different alphabets are significantly different for each type of yogurt
[AB]Means in the same row with different alphabets are significantly different for a particular day of storage for each parameter

Table 6.13 Changes in viscoelastic properties (at 1.5 Hz) during storage for 28 d of control (NEPSY) and experimental (EPSY) low-fat yogurts at 4 °C.

Type of Yogurt	Period of Storage (Day)				
	1	7	14	21	28
Storage Modulus (Pa)					
NEPSY	366.67^{aA}	452.00^{aA}	677.33^{aA}	813.67^{abA}	1429.83^{bA}
EPSY	179.33^{aB}	242.17^{aB}	220.50^{aB}	317.67^{aB}	382.17^{aB}
Loss Modulus (Pa)					
NEPSY	100.12^{aA}	119.77^{aA}	176.48^{aA}	210.65^{abA}	369.67^{bA}
EPSY	49.45^{aB}	64.63^{aB}	57.72^{aB}	82.70^{aB}	99.23^{aB}
Damping Factor (tan δ)					
NEPSY	0.274^{aA}	0.265^{bA}	0.261^{bdA}	0.259^{bcdA}	0.259^{bcdA}
EPSY	0.277^{aA}	0.268^{bB}	0.262^{bcA}	0.261^{bcB}	0.259^{bcA}

Values are the means of six observations
NEPSY = control yogurt prepared from skim milk standardized to 12% total solids containing 3% inulin and non-EPS producing strain of *S. thermophilus* 1342; EPSY = Yogurt prepared from skim milk standardized to 12% total solids containing 3% inulin and EPS producing strain of *S. thermophilus* 1275
[abcd]Means in the same row with different alphabets are significantly different for each type of yogurt
[AB]Means in the same column with different alphabets are significantly different for a particular day of storage for each parameter

Table 6.14 Flow behaviour (predicted by the Herschel-Bulkley model) and hysteresis loop area of control (NEPSY) and experimental (EPSY) low-fat yogurts during storage for 28 d at 4 °C.

Storage Period (day)	Rheological parameters									
	σ_0 (Pa)		k (Pa.s)		n		R^2		Hysteresis (Pa/s)	
	NEPSY	EPSY	NEPSY	EPSY	NEPSY	EPSY	NEPSY	EPSY	NEPSY	EPSY
1	8.00^{aA}	5.25^{aA}	2.06^{aA}	1.22^{aA}	0.62^{aA}	0.73^{aA}	0.990	0.983	260.91^{aA}	134.08^{aA}
7	11.65^{acA}	6.88^{acB}	1.40^{aA}	1.30^{aA}	0.77^{bA}	0.73^{aA}	0.990	0.985	295.41^{aA}	207.36^{aA}
14	15.28^{bcdA}	6.84^{acB}	2.33^{abA}	1.22^{aA}	0.65^{abA}	0.75^{acA}	0.985	0.986	486.46^{bA}	189.49^{aB}
21	18.06^{bdA}	9.31^{bcdB}	3.18^{abA}	1.07^{aB}	0.74^{abA}	0.81^{acA}	0.983	0.985	515.27^{bA}	253.84^{aB}
28	27.84^{bA}	11.06^{bdB}	4.01^{bcA}	0.94^{aB}	0.66^{abA}	0.86^{bcB}	0.985	0.985	897.06^{bcA}	298.08^{baB}
SEM	1.41		0.53		0.05				51.65	

Values are the means of six observations; SEM = standard error of means

NEPSY = control yogurt prepared from skim milk standardized to 12% total solids containing 3% inulin and non-EPS producing strain of *S. thermophilus* 1342; EPSY = yogurt prepared from skim milk standardized to 12% total solids containing 3% inulin and EPS producing strain of *S. thermophilus* 1275

[abc]Means in the same row with different alphabets are significantly different for each type of yogurt

[AB]Means in the same column with different alphabets are significantly different for a particular day of storage for each parameter

Table 6.15 Flow behaviour (predicted by the Herschel-Bulkley model) and hysteresis loop area of control (NEPY) and experimental (EPY) low-fat probiotic yogurts during storage at 4 °C for 28 d.

Storage Period (day)	Rheological parameters									
	σ_0 (Pa)		*k* (Pa.s)		n		R^2		Hysteresis (Pa/s)	
	NEPY	EPY	NEPY	EPY	NEPY	EPY	NEPY	EPY	NEPY	EPY
1	7.12^{aA}	7.83^{aA}	1.87^{aA}	1.97^{aA}	0.69^{aA}	0.69^{aA}	0.99	0.99	249.16^{aA}	281.95^{aA}
7	14.18^{bA}	7.57^{aB}	1.72^{aA}	1.21^{aA}	0.77^{aA}	0.77^{aA}	0.99	0.98	413.35^{aA}	217.17^{aB}
14	12.47^{bdA}	7.28^{aB}	2.23^{aA}	2.00^{acA}	0.71^{aA}	0.70^{aA}	0.99	0.99	370.08^{acA}	274.29^{acA}
21	17.50^{beA}	11.50^{acB}	2.68^{acA}	2.48^{abcA}	0.71^{aA}	0.70^{aA}	0.99	0.99	587.26^{badA}	441.43^{abcdA}
28	21.61^{bceA}	14.42^{bcB}	3.52^{bcA}	1.71^{acB}	0.71^{aA}	0.82^{baA}	0.99	0.99	771.33^{bA}	499.84^{bdB}
SEM	1.61		0.38		0.04				65.08	

NEPY = control yogurt prepared from skim milk standardized to 12% total solids containing 3% Orafti HP, probiotics and non-EPS producing strain of *S. thermophilus* 1342; EPY = experimental yogurt prepared from skim milk standardized to 12% total solids containing 3% Orafti HP, probiotics and EPS producing strain of *S. thermophilus* 1275

^{abcde}Means in the same column with different alphabets are significantly different ($P < 0.05$) for each type of yogurt

^{AB}Means in the same row with different alphabets are significantly different ($P < 0.05$) for a particular day of storage for each parameter

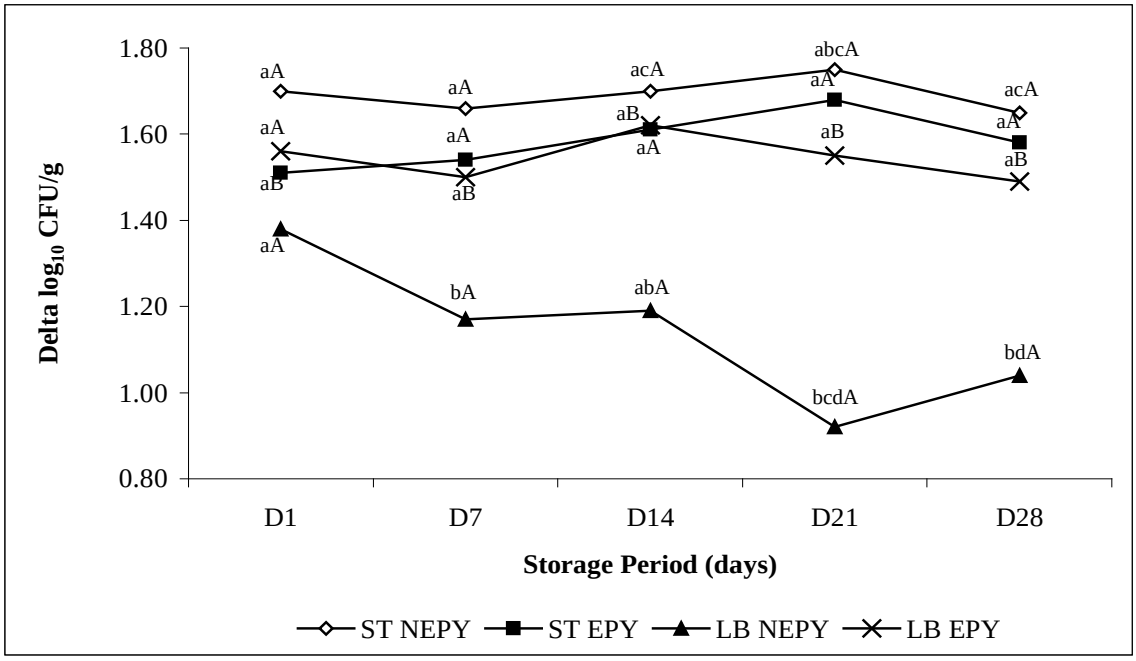

Figure 6.1 Changes in viability of yogurt starters (Δlog_{10} CFU/g) in control (NEPY) and experimental (EPY) low-fat probiotic yogurts during storage at 4 °C for 28 d. ST = *S. thermophilus* 1275 and 1342; LB = *L. delbrueckii* ssp. *bulgaricus* 1368. SEM for *S. thermophilus* = 0.04 and for *L. delbrueckii* ssp. *bulgaricus* = 0.07. (^{abcd}Means with different alphabets are significantly different within each type of yogurt; ^{AB}Means with different alphabets are significantly different between each type of yogurt for a particular day of storage)

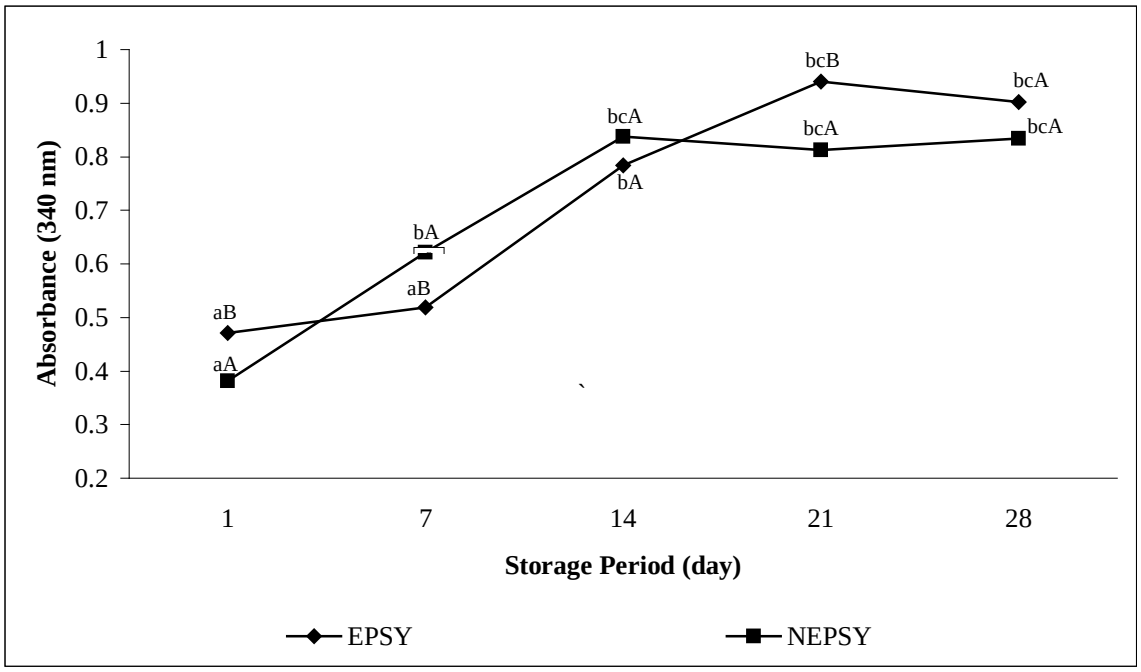

Figure 6.2 Changes in proteolysis (ΔA_{340}) of control (NEPSY) and experimental (EPSY) low-fat yogurts during storage for 28 d at 4 °C. NEPSY = control yogurt prepared from skim milk standardized to 12% total solids containing 3% inulin and non-EPS producing strain of *S. thermophilus* 1342; EPSY = yogurt prepared from skim milk standardized to 12% total solids containing 3% inulin and EPS producing strain of *S. thermophilus* 1275 (^{abc}Means with different alphabets are significantly different within each type of yogurt; ^{AB}Means with different alphabets are significantly different between each type of yogurt for a particular day of storage).

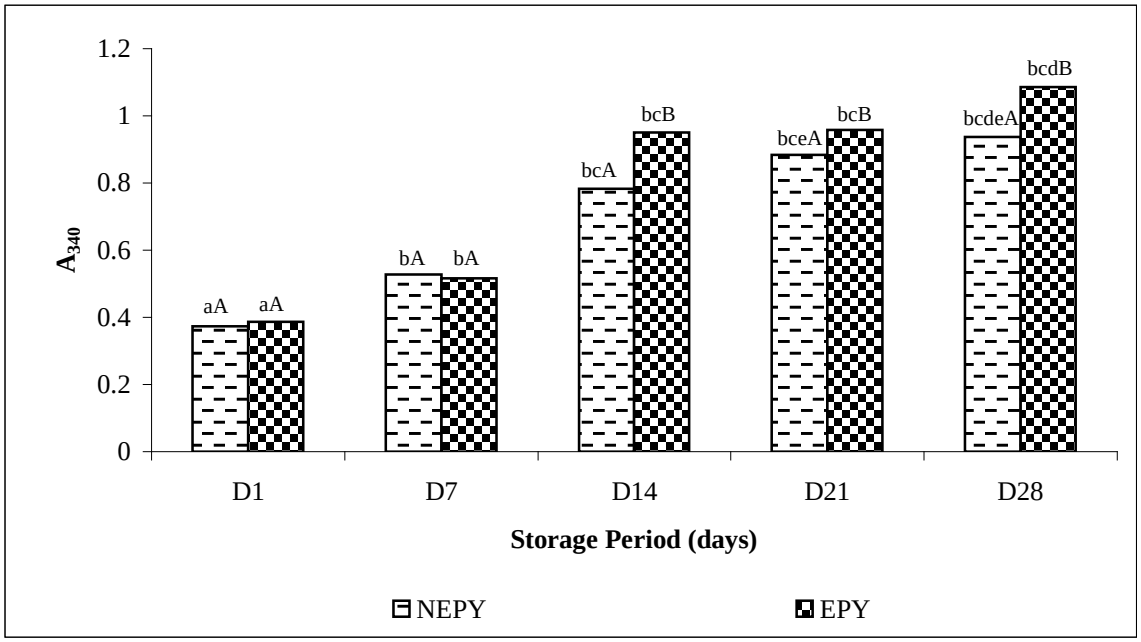

Figure 6.3 Changes in extent of proteolysis (A_{340}) in control (NEPY) and experimental (EPY) low-fat probiotic yogurts during storage at 4 °C for 28 d. SEM = 0.038. ([abcd]Means with different alphabets are significantly different within each type of yogurt; [AB]Means with different alphabets are significantly different between each type of yogurt for a particular day of storage)

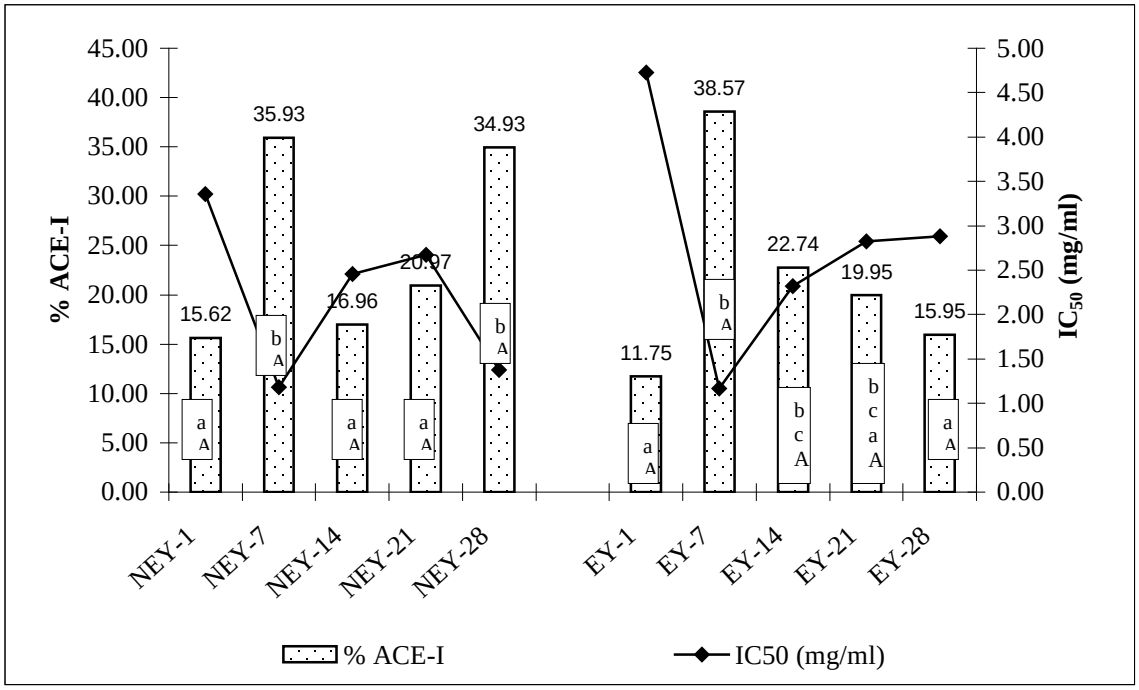

Figure 6.4 Changes in ACE-inhibition (%) and IC_{50} (mg/ml) values of control (NEY) and experimental (EY) low-fat yogurts during storage at 4 °C for 28 d. Subscript 1 to 28 following the yogurt types indicates storage period d 1 to 28. (^{abc}Means with different alphabets are different within each type of yogurt; ^{AB}Means with different alphabets are different between each type of yogurt for a particular day of storage)

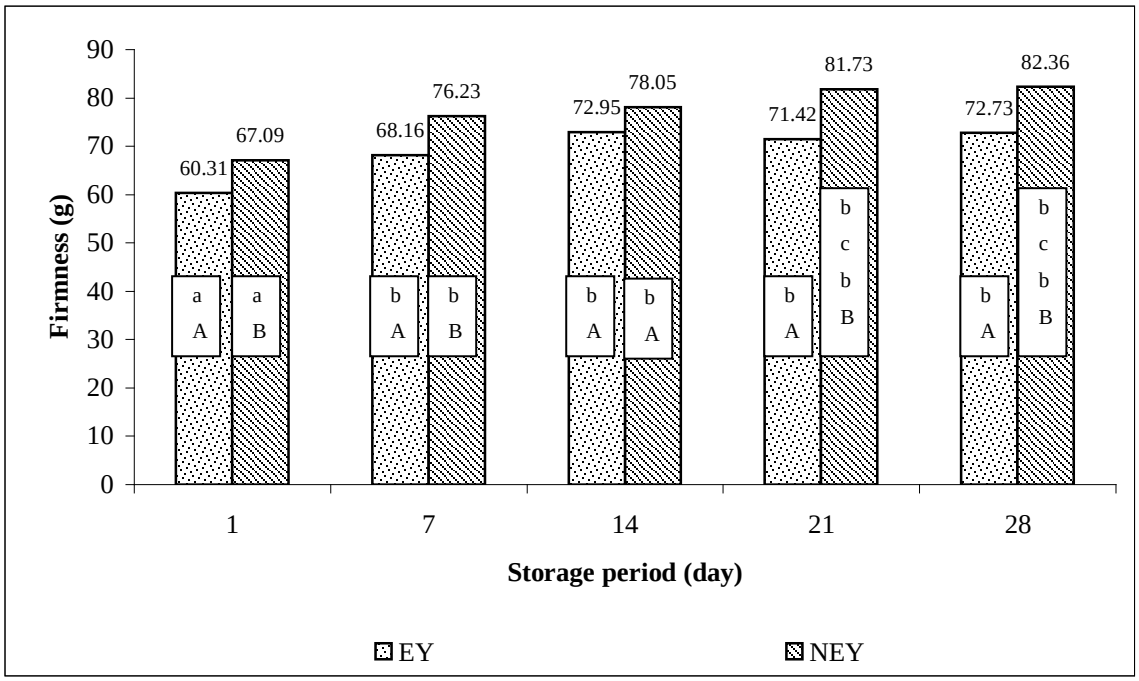

Figure 6.5 Changes in firmness (g) of control (NEY) and experimental (EY) low-fat yogurts during storage at 4 °C for 28 d. (^{abc}Means with different alphabets are significantly different within each type of yogurt; ^{AB}Means with different alphabets are significantly different between each type of yogurt for a particular day of storage)

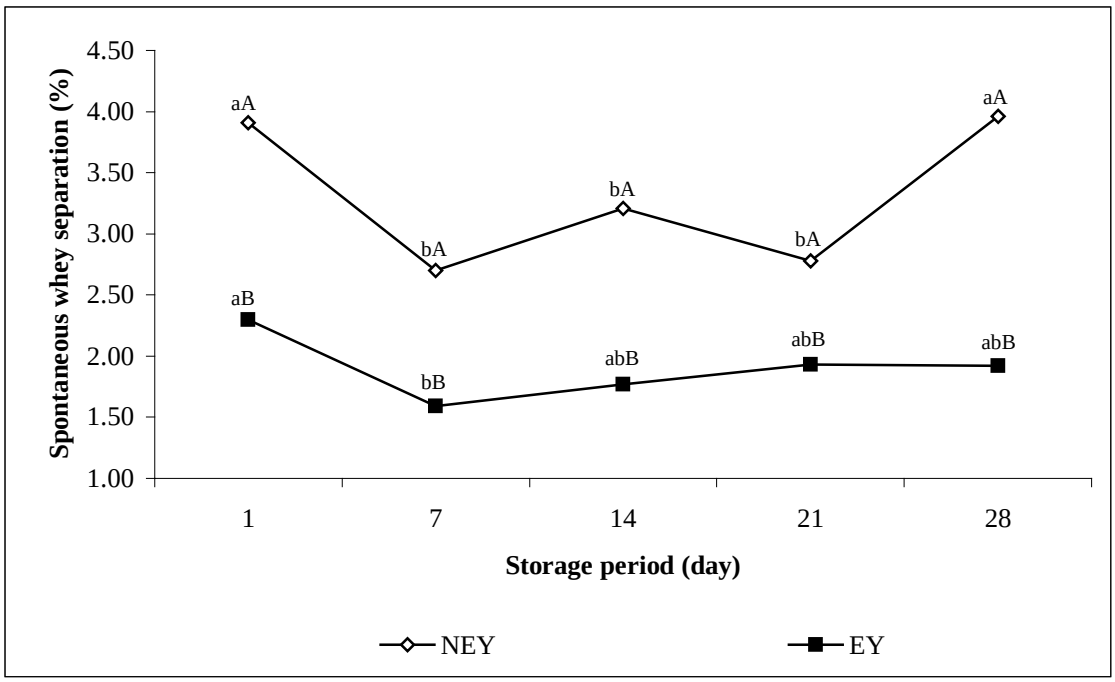

Figure 6.6 Changes in spontaneous whey separation (%) during storage of control (NEY) and experimental (EY) low-fat yogurts at 4 °C for 28 d. (^{ab}Means with different alphabets are significantly different within each type of yogurt; ^{AB}Means with different alphabets are significantly different between each type of yogurt for a particular day of storage).

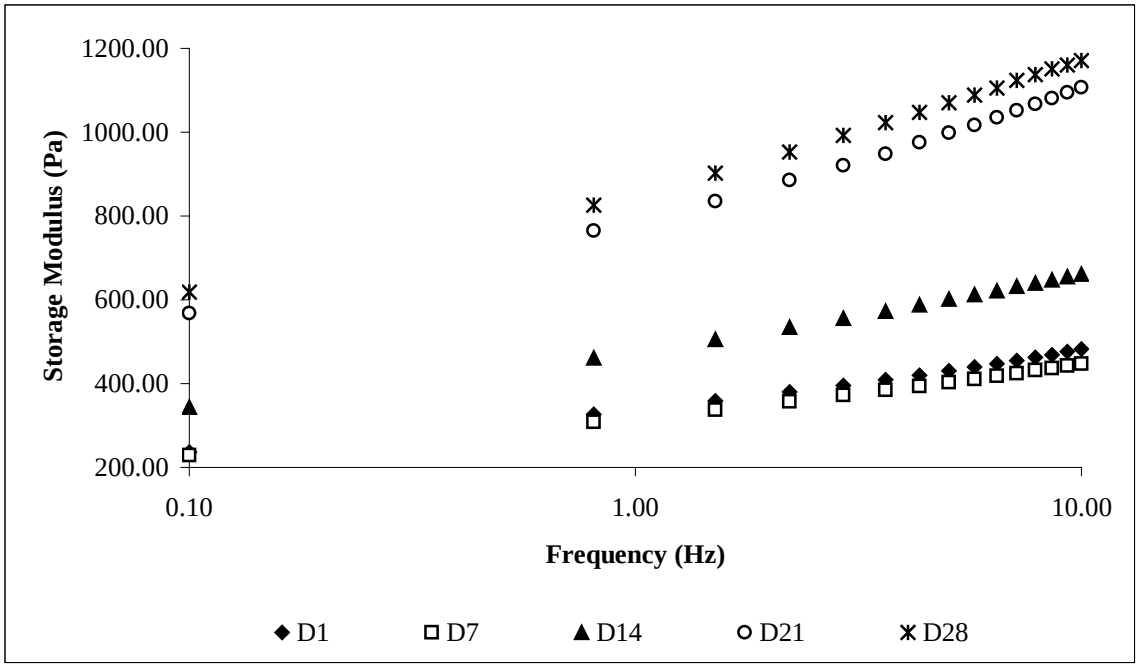

Figure 6.7 Storage modulus of NEY during storage at 4 °C for 28 d, as a function of oscillatory frequency, carried out at 5 °C. Reported data are means of six observations.

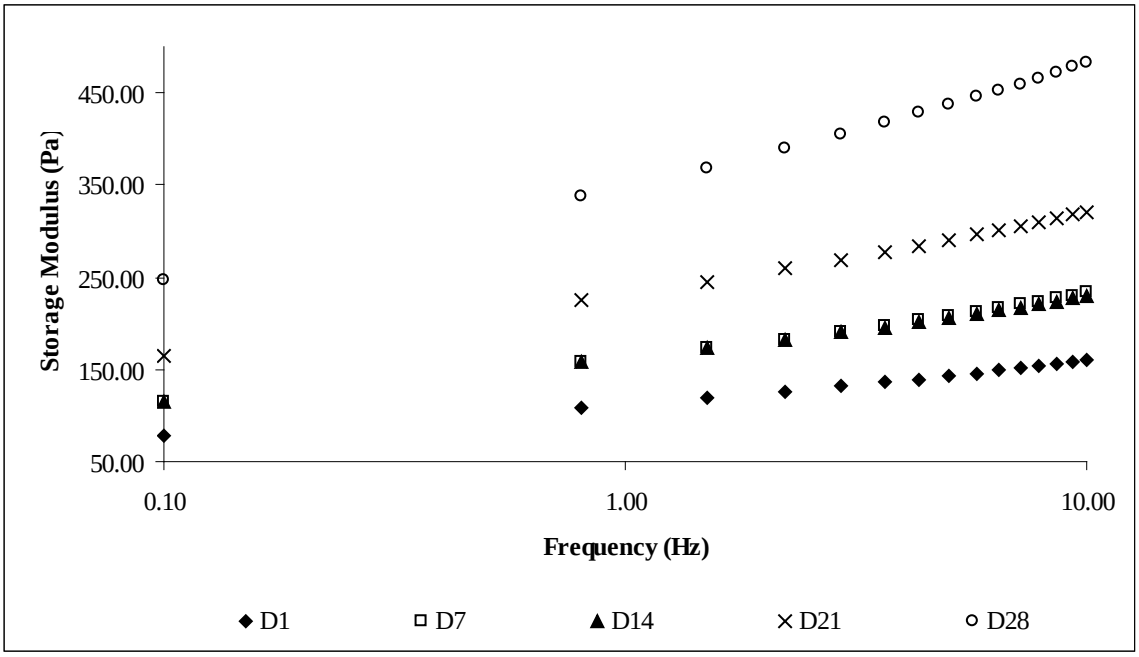

Figure 6.8 Storage modulus of EY during storage at 4 °C for 28 d, as a function of oscillatory frequency, carried out at 5 °C. Reported data are means of six observations.

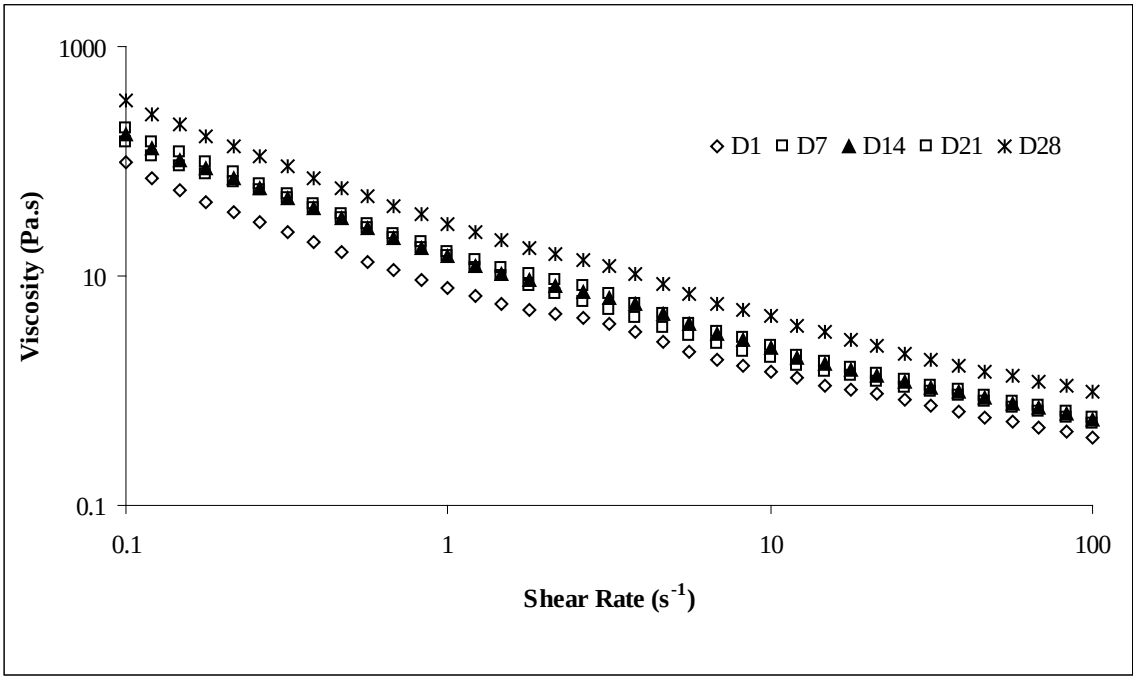

Figure 6.9 Changes in viscosity with shear rate in control low-fat yogurt during storage at 4 °C for 28 d. Control yogurt prepared from skim milk standardized to 12% total solids containing 3% inulin and non-EPS producing strain of *S. thermophilus* 1342.

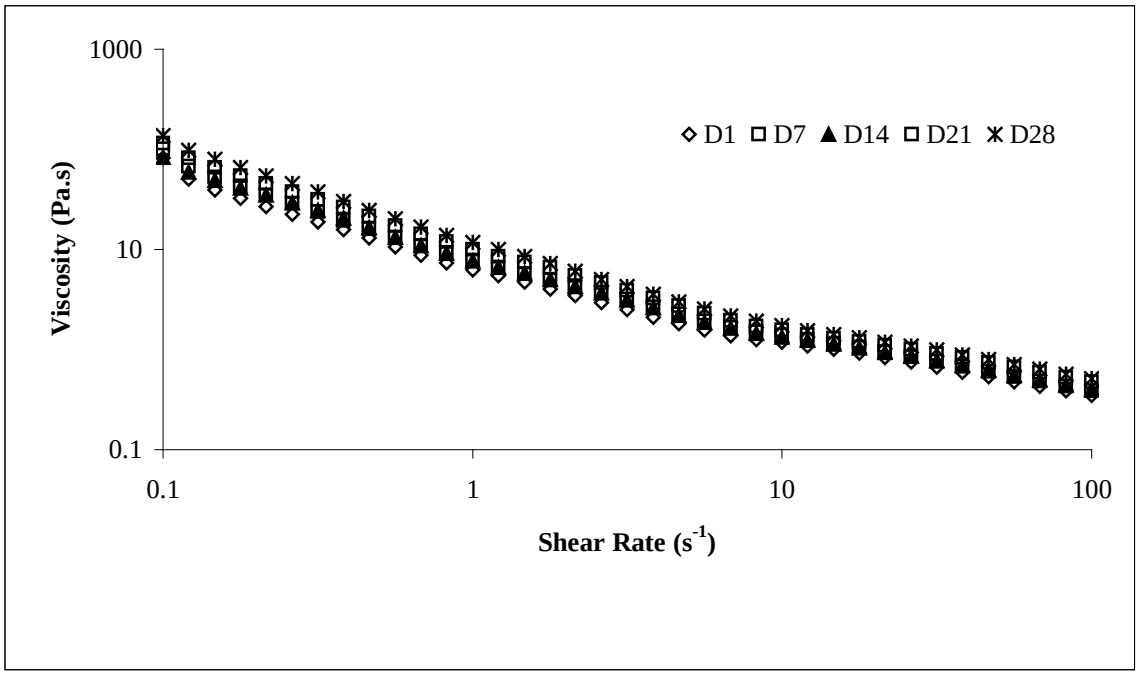

Figure 6.10 Changes in viscosity with shear rate in experimental low-fat yogurt during storage for 28 d at 4 °C. Experimental yogurt prepared from skim milk standardized to 12% total solids containing 3% inulin and EPS producing strain of *S. thermophilus* 1275.

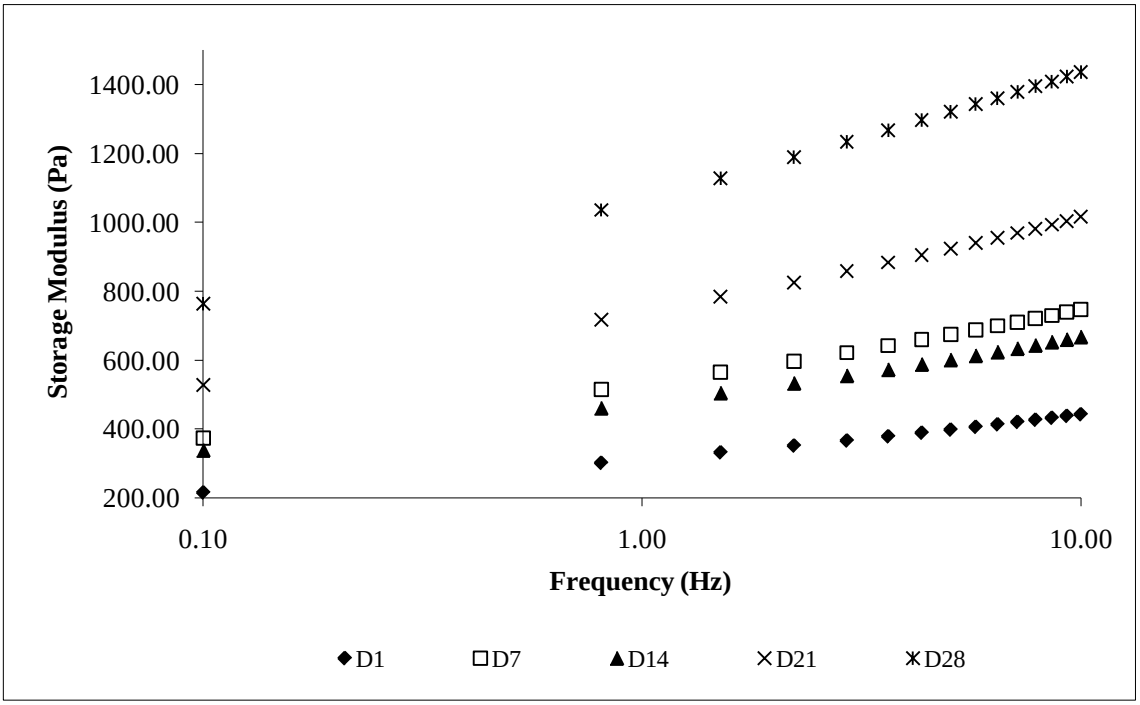

Figure 6.11 Storage modulus of NEPY measured at 1.5 Hz, during storage at 4 °C for 28 d, as a function of oscillatory frequency, carried out at 5 °C. Reported data are means of six observations.

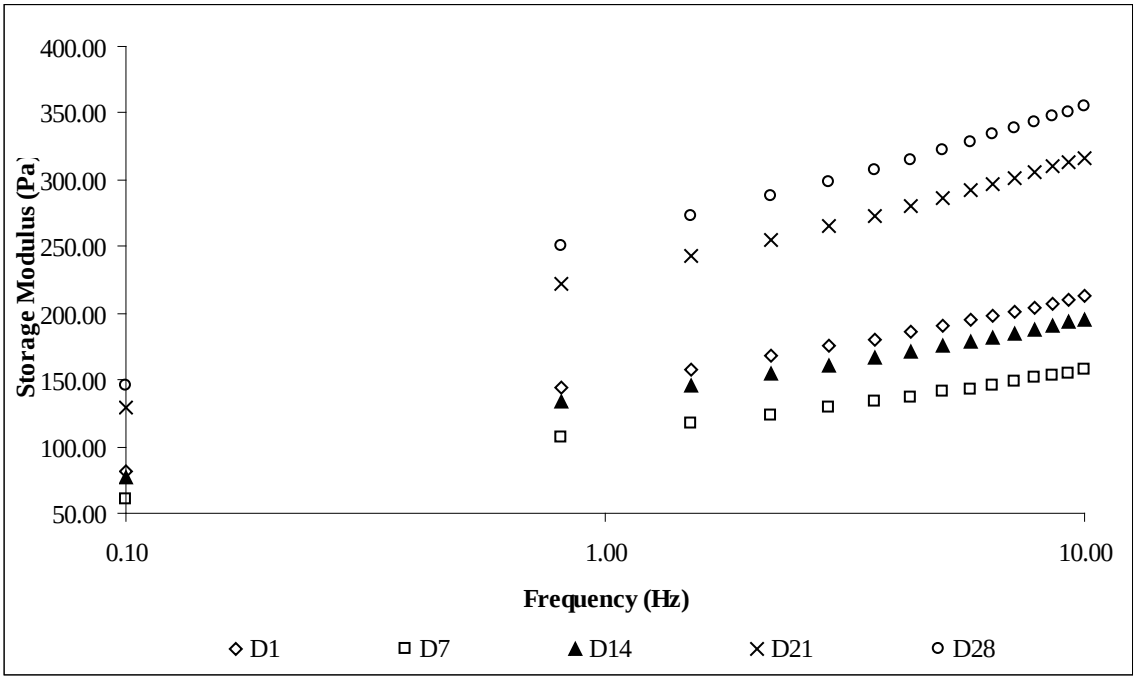

Figure 6.12 Storage modulus of EPY measured at 1.5 Hz, during storage at 4 °C for 28 d, as a function of oscillatory frequency, carried out at 5 °C. Reported data are means of six observations.

7.0 Yogurt Can Beneficially Affect Blood Contributors of Cardiovascular Health Status in Hypertensive Rats

A version of this chapter has been submitted for publication.

Ramchandran, L., and Shah, N. P. 2010. Yogurt can beneficially affect blood contributors of cardiovascular health status in hypertensive rats. Dairy Science and Technology (earlier known as Le Lait) (awaiting editor's decision).

7.1 Introduction

Coronary heart disease (CHD) is a type of blood vessel disease that continues to be the most common and serious form of cardiovascular disease (CVD) and the leading cause of death worldwide. Diets rich in low-fat dairy products are part of the current dietary advice to reduce risk of CVD. Consequently there has been an increased focus on improving diet and lifestyle as a strategy for CVD risk reduction (Erdmann et al., 2008).

One of the major independent risk factors for CVD is elevated blood pressure (BP). Angiotensin I-converting enzyme (ACE) plays a crucial role in the regulation of blood pressure. In recent years some food proteins have been identified as sources of ACE-inhibitory peptides and are currently the best known class of bioactive peptides (Fitzgerald et al., 2004). These peptides have received considerable attention for their effectiveness in both the prevention and the treatment of hypertension. Numerous studies in spontaneously hypertensive rats (SHR) and human volunteers have been performed to determine the antihypertensive effect of food-derived ACE-inhibitors. Spontaneously hypertensive rat is an animal model of essential (or primary) hypertension, used to study cardiovascular disease. These *in vivo* studies have demonstrated that several ACE-inhibitory peptides reduce blood pressure, either after intravenous or oral administration (Yuan and Kitts, 1993; Yamamoto et al., 1999; Kawase et al., 2000; Sipola et al., 2002; Seppo et al., 2003). It was also observed that the peptides being studied have little or no effect on blood pressure of normotensive subjects suggesting that they exert no acute hypotensive effect. Therefore, ACE-inhibitory peptides could represent a low-cost alternative treatment for hypertension and could be applied as initial treatment in mildly hypertensive individuals or as supplemental treatment with the added benefit of no harmful side effects (Seppo et al., 2003). These peptides are known to be produced during the manufacture of fermented milk products such as yogurt as a consequence of the proteolytic activity of the starter organisms and also the probiotics included in such products. The formation of such peptides may be further promoted by the consumption and digestion of these products. Not all peptides possessing high ACE-inhibition activity *in vitro* show strong antihypertensive activity *in vivo* (Maeno et al., 1996). Therefore it is assumed that these peptides may influence blood pressure (BP) by mechanisms other than the established ACE-inhibition (Yamamoto et al., 1999). A few studies have reported the antihypertensive effect of fermented products such as yogurt on

animals or human volunteers (Seppo et al., 2003; Mizushima et al., 2004; Tuomilehto et al., 2004) while some have reported the antihypertensive effect of specific isolated peptides (Nakamura et al., 1995b; Matar et al., 2003).

Another important risk factor for the genesis of various CVDs is an unfavourable profile of blood lipids. Many studies have found a positive correlation between hypercholesterolemia and/or hypertriglyceridemia and the likelihood for developing CVD. Yogurt and other fermented milk products have been reported to contain some substances that lower serum cholesterol (Akalin et al., 1997; Beena and Prasad, 1997; Pereira and Gibson, 2002; Xiao et al., 2003). Kawase et al. (2000) observed significant increase in high density lipoprotein (HDL)-cholesterol level and decrease in triglyceride (TG) and atherogenic index (AI) in the blood serum of rats fed milk fermented with *L. casei* EMC0409 and *S. thermophilus* TMC1543. Such reductions were accompanied by a decrease in systolic BP of human volunteers consuming the fermented milk. It is also reported that prebiotics such as fructooligosaccharides, inulin and oligofructose, showed convincing lipid-lowering effects, but only at high dose levels (50-200 g/kg) in animals (St-Onge et al., 2000; Mortenson et al., 2002; Pereira and Gibson, 2002). Kießling et al. (2002) concluded that long-term daily consumption of symbiotic yogurt, containing *S. thermophilus*, *L. lactis*, *L. acidophilus* 145, *B. longum* 913 and 1% oligofructose did not lower the total and LDL-cholesterol in healthy women but increased the serum concentration of HDL-cholesterol and led to the desired improvement of LDL/HDL ratio. Additionally, exopolysaccharides (EPS) produced by the starter lactic acid bacteria are also known to have a beneficial effect on cholesterol metabolism (Nakajima et al., 1992). A study on the effects of kefiran, an EPS produced by *L. kefiranofaciens*, in animals demonstrated that kefiran suppressed increase of BP and reduced the serum cholesterol levels in SHR rats (Maeda et al., 2004).

Our earlier experiments have shown that low-fat yogurt and probiotic yogurt, both containing inulin, exhibited enhanced ACE-inhibitory activity *in vitro* than low-fat yogurts without either inulin or probiotics (Ramchandran and Shah, 2009c, 2010). This study was in continuation of our previous work with the specific aim of evaluating *in vivo*, during an 8 wk feeding period, the antihypertensive effect of the low-fat yogurts as well as their influence on the serum lipid profile of SHR including TG, total cholesterol, HDL-cholesterol, and LDL-cholesterol. The weight gain and feed intake of the animals was also recorded during this period. The ACE-inhibitory activity of the yogurts, their freeze dried forms as well as the corresponding feeds were also monitored.

7.2 Materials and Methods

7.2.1 Yogurt preparation and animal diets

Two types of yogurts were selected for the study based on our earlier experiments (Ramchandran and Shah, 2009c, 2010). The yogurts were prepared in bulk for incorporating into the diet of the experimental animals. Skim milk (20 L per batch) was preheated to 60 °C in covered stainless steel vats and fortified with skim milk powder (3%, w/v) followed by the addition of 3% Beneo HP® at 80 °C (the addition was shown to improve ACE-inhibition activity in our earlier experiment, Ramchandran and Shah, 2008b). The yogurt mix was then heat treated to 85 °C and held at that temperature for 30 min before being cooled in a chilled water tank (4 °C) to 45 °C. The yogurt milk mix was divided into two batches. One batch (Y) was inoculated with yogurt starters (*S. thermophilus* 1275 and *L. delbrueckii* ssp. *bulgaricus* 1368) and the other (PY) with yogurt starters and probiotics, *L. acidophilus* 4461, *L. casei* 15286 and *B. longum* 5022, each at 1% (w/v). The inoculated mixes were fermented at 42 ± 1 °C until the pH dropped to 4.5. The yogurt containers were then transferred into a walk-in refrigerator maintained at 4 °C. After 1 wk of refrigeration (shown to have higher ACE-inhibitory activity than fresh yogurts in our earlier experiments, unpublished data; Chapters 9.0 and 10.0), the yogurts were freeze dried (Biotech Freeze Drying, Knoxfield, Melbourne, Vic) and sent for formulation of experimental diets (Specialty Feeds, Perth, Western Australia). The control and experimental diets for the experiment were formulated as indicated in Table 7.1. The amount of skim milk powder in the formulated diets was limited by the process of pelleting. It was observed that quantities above 44.8% hindered the process of pellet formation due to the caking property of the lactose in skim milk powder. Consequently, the quantity of freeze dried yogurts (FDY and FDPY) that were incorporated in the experimental diets (Feed-Y and Feed-PY, respectively) in lieu of skim milk powder of the control diet (Feed-C) was also limited. All the diets were pelleted and stored at 4 °C until use.

7.2.2 Experimental animals

The protocol of the experiment was approved by the Animal Experimentation Ethics Committee, Victoria University (AEETH 10/07). Eighteen male SHR (purchased from Animal Resources Centre, Western Australia), 14 weeks old and weighing 270-290 g on arrival were housed in pairs in a temperature controlled environment (20-22 °C) with a 12 h light-dark cycle. Following a 1 week adaptation period in which the animals were fed a rat

chow diet, the animals were randomly divided into three dietary groups (n = 6 per group) and housed individually to receive the control and experimental diets for a period of 8 week: the control group (RC) was fed the control skim milk diet while the experimental groups (RY and RPY) were fed the diets supplemented with yogurt (Feed-Y) and probiotic yogurt (Feed-PY), respectively. Feed and water were provided *ad libitum* throughout the study. The feed consumption and body weight gain of the rats were recorded weekly. At the end of the experimentation period the rats were euthanized by overdosing (50 mg/kg body weight) with sodium pentobarbital and blood samples were drawn into sterile eppendorfs by cardiac puncture of the animals for analysis of serum lipid profile.

7.2.3 Blood pressure (BP) measurements

Systolic and diastolic BP of the rats were measured every 2 wk using a small animal tail noninvasive blood pressure system (BIOPAC Systems, Inc, CA). Before the measurement, the animals were held in a restrainer and warmed to 30 ± 1 °C to make the pulsations of the tail artery detectable. At least five measurements were obtained on each animal and three closest measurements were considered for the statistical analysis. The measurements were obtained using MP100 data acquisition system and AcqKnowledge 3.9.1 software provided by BIOPAC systems.

7.2.4 Serum lipid profile

The blood samples withdrawn were allowed to coagulate by standing the samples at room temperature (20-22 °C) for 30 min followed by centrifugation (Eppendorf 5415C centrifuge, Crown Scientific, Melbourne, Vic, Australia) at 2000 × g for 20 min at 4 °C. The serum thus obtained was analysed for TG, total cholesterol and HDL-cholesterol using test kits (Thermo Fischer Scientific, Melbourne, Australia). The LDL-cholesterol was calculated by the formula:

LDL-cholesterol = (total cholesterol – HDL-cholesterol) – (triglyceride/5)

The atherogenic index (AI) was also calculated by the formula:

AI = (Total cholesterol – HDL-cholesterol)/HDL-cholesterol

7.2.5 *In vitro* ACE-inhibition (%) and IC_{50} (mg/ml)

The ACE-inhibitory activity of the two types of yogurts, Y and PY, the corresponding freeze dried samples, FDY and FDPY as well as Feed-Y and Feed-PY were also analysed. The supernatant from the yogurt samples, refrigerated for 1 wk, were prepared

as described in Section 6.2.3. The extracts of the freeze dried yogurts and feeds were prepared by reconstituting the samples (to the same level of TS as the yogurt) in milli Q water followed by centrifugation. All the supernatants thus obtained were filtered through 0.45 µm syringe membrane filter and stored at - 20 °C until assayed. The ACE-inhibitory activity and IC_{50} values were obtained by the method described in Section 3.2.6.

7.2.6 Statistical analysis

Two-way analysis of variance was used to determine variation within and between dietary period and diets for weight gain, feed intake, systolic and diastolic BP. One-way analysis of variance (completely randomized block design) was used to determine variations in ACE-inhibition and serum lipid profile. The least significant difference test for mean separation was used to determine the statistical significance between the different types of yogurts and diets of the same variable as outlined by SAS (SAS, 2003). All data are reported as means and standard deviations of means.

7.3 Results and Discussion

7.3.1 Feed intake and gain in body weight

The average weekly feed intake (g) of each dietary group of experimental rats and the corresponding gain in body weight is given in Table 7.2. The feed intake was similar for 7 wk of the study period for all the dietary groups of SHR. The feed intake reduced ($P < 0.05$) in the control group (RC) during wk 8 of the study as compared to that of the experimental diet groups for the last week. Intermittent period of reduced feed intake was observed in all the groups during the 8 wk period. The weight gain of the three dietary groups of rats was steady for the first 4 wk and thereafter the weight gain varied for different groups, showing periods of increase and decrease. These variations did not match the variations in feed intake. The total weight gain for the 8 wk period (body weight at wk 8 less body weight at the start) was in the order RC (90 g) > RPY (85.7g) > RY (78.7g), indicating that the overall weight gains were lesser in the groups fed yogurt containing diets than that in the group fed skim milk diet.

7.3.2 Antihypertensive effect – *in vitro* and *in vivo*

The *in vitro* ACE-inhibition (%) and the IC_{50} (mg/ml) values of the prepared yogurts (Y and PY) after 1 wk of storage at 4 °C, and the corresponding freeze dried yogurts (FDY

and FDPY) and the diets containing yogurts (Feed-Y and Feed-PY) is presented in Table 7.3. The yogurt milk bases and the skim milk diet did not show any ACE-inhibitory activity. In general, the ACE-inhibitory activity decreased ($P < 0.05$) when the yogurts were freeze dried but increased ($P < 0.05$) in the corresponding pelleted diets. This indicates that post-fermentation processes significantly affected the ACE-inhibitory activity of the product. Considering that the diets were supplemented upto 44.5% with freeze dried yogurts, the increase in ACE-inhibitory activities were appreciable ($P < 0.0.5$) as indicated by the lowered IC_{50} values as compared to the corresponding yogurts. However, the extent of increase in the antihypertensive effect was similar in both the yogurts (an overall decrease of IC_{50} 0.58-0.59 mg/ml). On the whole, PY, FDPY and Feed-PY had significantly higher ACE-inhibitory activity than the corresponding products without probiotics (Y, FDY and Feed-Y, respectively) indicating that inclusion of probiotics can be beneficial.

The BP values at the start of feeding (0 d averaging 150/94 mm Hg) were taken as the baseline for each group of rats to measure the change in BP with time. At the start of the study the systolic BP (Figure 7.1) was similar in all groups. The systolic BP of RC did not change throughout the study period while those of RY and RPY showed a biphasic change in BP – first a decreasing phase (4 wk) and then a steady unchanged phase (4 wk for RY and 2 wk for RPY). However, comparison of the pattern of decrease among the dietary groups showed that the change in systolic BP was significant ($P = 0.0008$) for RPY at wk 2 than for RC and RY after which the change was similar for RY and RYP for the rest of the period, except at wk 8 when RPY showed an increase in systolic BP resulting in a value higher ($P = 0.03$) than PY but lower ($P = 0.0002$) than the control. Among the dietary groups, the change in systolic BP taking that of RC as the baseline, for wk 2, 4, 6 and 8 were -1.34, -8.8, -8.18 and -9.46 mm Hg in RY while in RPY they were -4.29, -8.1, -9.44 and -6.91 mm Hg, respectively. In general ACE-inhibitory peptides that have been found to have antihypertensive activity in SHR have IC_{50} values lower than 150 µM (Matar et al., 2003). Thus, despite relatively low ACE-inhibitory activity of the feeds (Table 7.3), a significant antihypertensive effect was observed in SHR fed the diets containing the yogurts. Also, the higher ACE-inhibition of Feed-PY did not translate into higher reduction in systolic BP of SHR group RPY in comparison to those of group RY. This may be due to production of more ACE-inhibitory peptides during digestion of the feeds than those in the actual feed or due to an alternative mechanism of exerting antihypertensive effects. Vermeirssen et al. (2003) have indicated that only those ACE-inhibitors that are not affected by the action of angiotensin-II and gastrointestinal enzymes or those that are converted to stronger ACE-

inhibitors during digestion can exert antihypertensive effects *in vivo*. Yamamoto et al. (1999) also observed an antihypertensive effect (-29.6 mm Hg) in SHR orally fed the whey of a yogurt like product fermented with *L. helveticus* CPN4, which was attributed to the presence of a dipeptide Tyr-Pro. However, this dipeptide did not have a low ACE-inhibitory activity, suggesting alternate mechanism of exerting antihypertensive effect. Sipola et al. (2002) reported that a dose-related antihypertensive mechanism observed during long term treatment of SHR with fermented milks containing the tripeptides IPP and VPP was a result of raised plasma rennin activity. However, Yuan and Kitts (1993) did not observe any change in systolic BP of normotensive rats despite the reduced plasma TG and cholesterol levels in animals fed yogurt powder.

The pattern of changes in the diastolic BP (Figure 7.2) was almost similar to those of the systolic BP except that after a 4 wk period of decreasing diastolic BP, it increased, in all the groups, being more drastic in the control group. Yet, at the end of the feeding period, taking the diastolic BP of RC as baseline, the reduction in RY was -9.41 mm Hg while in RPY it was -13.84 mm Hg. It is evident from our results that feeding the diets containing yogurts, Feed-Y and Feed-PY, reduced the systolic and diastolic BP of the SHR rats as compared to the control diet and that Feed-Y had a better ability to maintain the lowered systolic but not diastolic BP than Feed-PY.

7.3.3 Serum lipid profile

The lipid profile of the blood serum of the SHR rats at the end of feeding is presented in Table 7.4. The level of TG was higher ($P < 0.05$) in RY than in RC while that in RPY was similar to that of RC. Consumption of fermented milks has been associated with decreased circulating cholesterol concentrations (St-Onge et al., 2000). Yuan and Kitts (1993) suggested that reductions in gastric emptying and peristalsis resulting from stimulation of the enterogastric reflex due to the increased acidity of the dietary components could have played a role in lowering absorption efficiency of dietary lipid which in turn resulted in reduction of both plasma TG as well as total cholesterol observed in animals fed yogurt powder. Also the decrease in LDL-cholesterol (Table 7.4) was higher ($P < 0.05$) in RPY than RY but the decrease in TG and total cholesterol was similar in RPY and RY. RPY exhibited a 35% reduction in LDL-cholesterol and 26.2% reduction in total cholesterol compared to a 30% and 23.3% reduction, respectively, in RY. This indicated a synergistic effect of yogurt starters and probiotics in lowering the LDL-cholesterol levels in SHR. A similar observation was made by Kawase et al. (2000) in 4 wk old Sprague-Dawley rats fed lyophilized

fermented milk with both *L. casei* TMC0409 and *S. thermophilus* TMC1543. The decrease in the level of serum total cholesterol of RY and RPY (Table 7.4), as compared to that in RC, resulted in lowered ($P < 0.05$) values of atherogenic index (AI) and ratio of total cholesterol to HDL-cholesterol while reduction of LDL-cholesterol values resulted in decreased ($P < 0.05$) ratio of LDL-cholesterol to HDL-cholesterol. The reduction in AI and ratios of total cholesterol to HDL-cholesterol and LDL-cholesterol to HDL-cholesterol indicated that the hypocholesterolemic effect accrued as a consequence of feeding Feed-Y and Feed-PY to SHR. However, there was no significant difference in the effects between the two experimental diets Feed-Y and Feed-PY. This suggested that the hypocholesterolemic effects were not affected by the inclusion of probiotics.

7.4 Conclusion

During feed preparation, the ACE-inhibitory activity decreased when the yogurts were freeze dried but increased in the corresponding pelleted diets. For the 8 wk feeding period, the total weight gain in three SHR groups was in the order RC (90 g) > RPY (85.7g) > RY (78.7g), indicating that the overall weight gains were lesser in the groups fed yogurt containing diets than skim milk containing diets. A definite antihypertensive effect was observed in SHR fed the diets containing the yogurts. At the end of the feeding period, taking the systolic and diastolic BP of control group as baseline, the reduction in group RY was -9.46 and -9.41 mm Hg while in the RPY group it was -6.91 and -13.84 mm Hg, respectively. It was concluded that feeding the diets containing yogurts reduced the systolic and diastolic BP of the SHR rats as compared to the control diet and that yogurt containing diet had a better ability to maintain the lowered systolic but not diastolic BP than probiotic yogurt containing diet. However, further experiments need to be conducted to see if individual probiotics can provide any beneficial effect. The lipid profile of RPY showed a 35% reduction in LDL-cholesterol and 26.2% reduction in total cholesterol and 30% and 23.3% reduction, respectively, in RY than those of RC suggesting a synergistic effect of yogurt starters and probiotics in lowering the LDL-cholesterol levels in SHR. The reduction in AI and ratios of total cholesterol to HDL-cholesterol and LDL-cholesterol to HDL-cholesterol indicated that the hypocholesterolemic effect accrued as a consequence of feeding Feed-Y and Feed-PY to SHR.

In summary, our results suggest that supplementation (~ 45%) of skim milk diet of SHR with freeze dried yogurt, with or without probiotics, aided in controlling weight gain, systolic and diastolic BP, serum TG, total cholesterol, and LDL-cholesterol without affecting the serum HDL-cholesterol levels. Therefore, it was concluded that it is possible to manufacture yogurt having multiple health benefits, having both serum lipid improvement and hypotensive effect on SHR. However, these effects need to be confirmed in human studies.

Table 7.1 Nutrient composition of diets.

Ingredient	Addition rate (g/100g)		
	Feed-C	Feed-Y	Feed-PY
Sucrose	10.00	10.00	10.00
Skim milk powder	44.48	0.00	0.00
Freeze dried yogurt (FDY)	0.00	44.48	0.00
Freeze dried probiotic yogurt (FDPY)	0.00	0.00	44.48
Canola oil	3.50	3.50	3.50
Cellulose	5.00	5.00	5.00
Starch	19.26	19.26	19.26
Dextrinised starch	15.50	15.50	15.50
DL-methionine	0.18	0.18	0.18
AIN-93-G-trace minerals	0.14	0.14	0.14
Calcium carbonate	0.06	0.06	0.06
Sodium chloride	0.18	0.18	0.18
Potassium sulphate	0.45	0.45	0.45
AIN-93-G-vitamins	1.00	1.00	1.00
Choline chloride 60% w/w	0.25	0.25	0.25

Table 7.2 Effect of feeding yogurt and probiotic yogurt containing diets on average weekly weight gain (g) and feed intake (g) of SHR.

Parameter (g)	Rat group	Week 1	2	3	4	5	6	7	8
Weight gain	RC	13.50±2.05^{aA}	15.83±3.73^{aA}	15.83±1.82^{aA}	11.33±1.36^{acA}	3.00±3.16^{bdA}	8.00±1.00^{abcdeA}	9.50±2.05^{abceA}	8.50±1.65^{abcdeA}
	RY	10.33±1.84^{aA}	12.33±1.56^{acA}	17.67±2.51^{bcA}	10.67±1.93^{acdA}	7.50±1.77^{acdeA}	10.50±1.91^{acdeA}	2.33±1.05^{befgB}	5.83±2.24^{adegA}
	RPY	11.50±4.01^{aA}	12.50±1.63^{aA}	15.50±3.23^{aA}	13.17±3.87^{acA}	5.50±2.39^{bdA}	9.33±1.71^{abcdA}	4.83±1.42^{bdAB}	8.33±1.09^{abcdA}
Feed intake	RC	98.75±3.40^{aA}	134.45±1.94^{bA}	133.38±3.48^{bA}	130.93±2.25^{bA}	134.83±2.45^{bA}	134.50±2.05^{bA}	129.73±1.77^{bA}	118.25±13.98$^{bc}_{A}$
	RY	91.17±2.76^{aA}	134.97±2.02^{bA}	137.62±2.99^{bA}	139.20±3.62^{bA}	138.18±4.32^{bA}	136.70±2.82^{bA}	127.17±2.87$^{bcd}_{A}$	135.52±4.04^{bdB}
	RPY	99.47±2.84^{aA}	136.42±4.00^{bA}	129.92±1.17^{bdA}	124.87±3.91$^{bcd}_{A}$	133.95±2.77^{bdA}	130.50±1.34^{bdA}	131.50±1.34^{bdA}	133.98±4.19^{bdB}

Values are the means of each group of rat (n = 6) ± standard deviation of means

[1] RC = control rat group fed skim milk diet; RY = rat group fed skim milk diet supplemented with freeze dried yogurt; RPY = rat group fed skim milk diet supplemented with freeze dried probiotic yogurt

[ab]Means in the same row with different alphabets are significantly different (P < 0.05) for each rat group

[AB]Means in the same column of each parameter with different alphabets are significantly different (P < 0.05) for the particular period of feeding

Table 7.3 *In vitro* ACE-inhibition and IC_{50} values of the yogurts, freeze dried yogurts and related feeds prepared.

Material		ACE-inhibition (%)	IC_{50} (mg/ml)
Yogurts	Y	24.48 ± 1.57^{cb}	3.02
	PY	38.03 ± 0.65^{a}	2.03
Freeze dried yogurts	FDY	21.05 ± 4.39^{c}	3.31
	FDPY	33.70 ± 9.44^{bac}	2.13
Supplemented diets	Feed-Y	26.47 ± 1.48^{bac}	2.43
	Feed-PY	36.04 ± 0.83^{ba}	1.45

Values are the means ± standard deviation of means
Y = low-fat yogurt; PY = low-fat probiotic yogurt; FDY = freeze dried yogurt; FDPY = freeze dried probiotic yogurt; Feed-Y = skim milk diet supplemented with FDY; Feed-PY = skim milk diet supplemented with FDPY
^{abc} t Grouping of the means. Means with different alphabets are significantly different ($P < 0.05$)

Table 7.4 Effect of feeding diets containing yogurt and probiotic yogurt on serum lipid profile of SHR.

Parameter	Rat Group		
	RC	RY	RPY
Triglycerides (mg/dL)	85.87±3.91[b]	98.45±3.45[a]	88.22±3.05[b]
Total cholesterol (mg/dL)	202.40±6.57[a]	155.31±3.95[b]	149.40±4.33[b]
HDL-cholesterol (mg/dL)	41.40±1.41[a]	39.13±0.91[a]	40.39±1.42[a]
LDL-cholesterol (mg/dL)	141.31±7.66[a]	118.16±4.64[b]	95.98±4.16[c]
Atherogenic index	3.98±0.28[a]	2.99±0.12[b]	2.75±0.17[b]
Total cholesterol/HDL-cholesterol	4.98±0.28[a]	3.99±0.12[b]	3.75±0.17[b]
Total cholesterol/HDL-cholesterol	4.98±0.28[a]	3.99±0.12[b]	3.75±0.17[b]
LDL-cholesterol/HDL-cholesterol	3.50±0.27[a]	2.54±0.11[b]	2.31±0.15[b]

Values are the means of each group of rat (n = 6) ± standard deviation of means
RC = control rat group fed skim milk diet; RY = rat group fed skim milk diet supplemented with freeze dried yogurt; RPY = rat group fed skim milk diet supplemented with freeze dried probiotic yogurt
[abc]Means with different alphabets are significantly different ($P < 0.05$) for each parameter

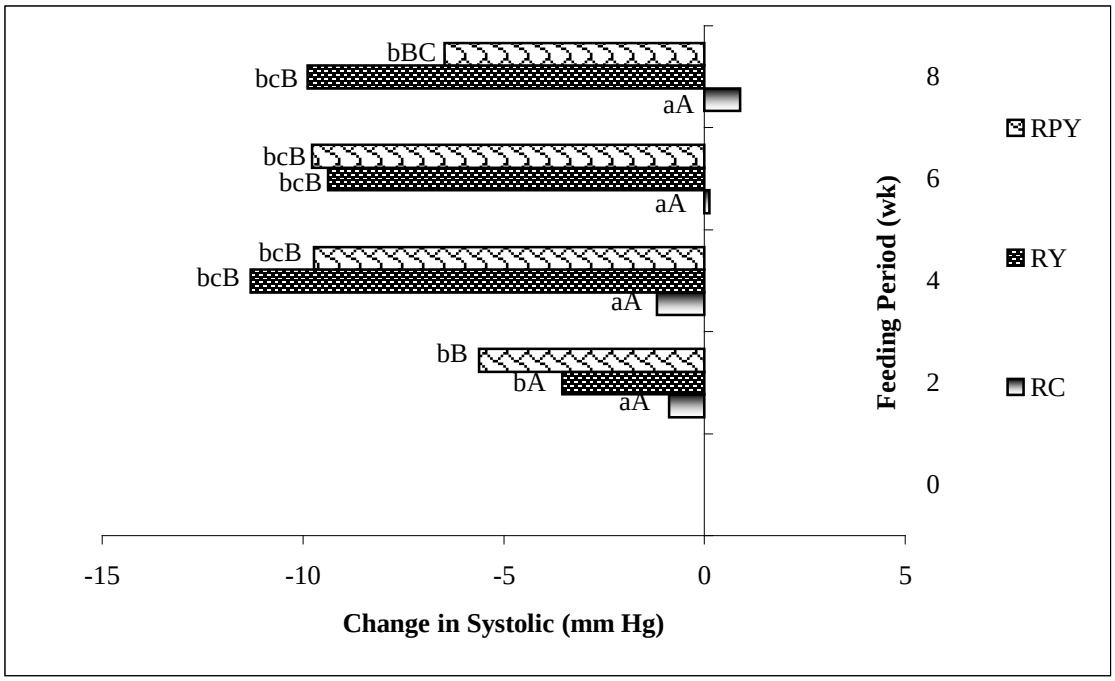

Figure 7.1 Changes in systolic blood pressure of SHR during feeding of control and experimental diets taking the values at start of feeding (0 d) as the baseline for each dietary group of SHR (n = 6). RC = control rat group fed skim milk diet; RY = rat group fed skim milk diet supplemented with freeze dried yogurt; RPY = rat group fed skim milk diet supplemented with freeze dried probiotic yogurt. ^{abc}Bars with different alphabets are significantly different ($P < 0.05$) for each rat group. ^{ABC}Bars with different alphabets are significantly different ($P < 0.05$) for the particular period of feeding.

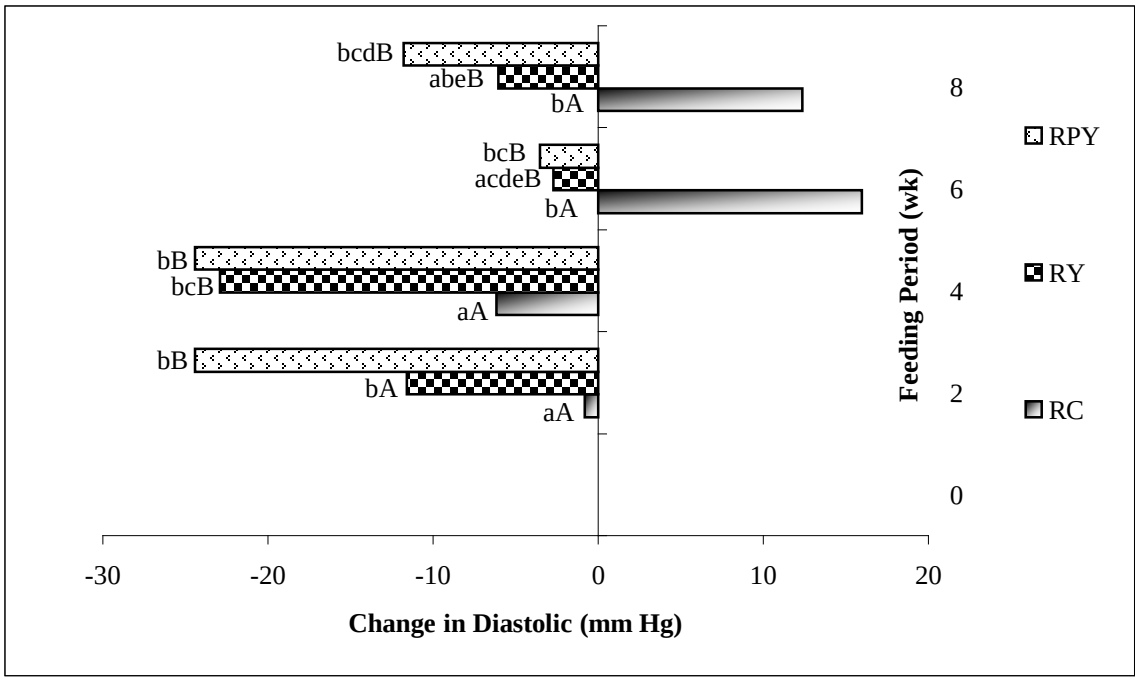

Figure 7.2 Changes in diastolic blood pressure of SHR during feeding of control and experimental diets taking the values at start of feeding (0 d) as the baseline for each dietary group of SHR (n = 6). RC = control rat group fed skim milk diet; RY = rat group fed skim milk diet supplemented with freeze dried yogurt; RPY = rat group fed skim milk diet supplemented with freeze dried probiotic yogurt. ^{ab}Bars with different alphabets are significantly different ($P < 0.05$) for each rat group. ^{AB}Bars with different alphabets are significantly different ($P < 0.05$) for the particular period of feeding.

8.0 Overall Conclusions

The nine selected strains of *S. thermophilus* 1275 and 285, *L. delbrueckii* ssp. *bulgaricus* 1092 and 1368, *L. casei* 2607 and 15286, *L. acidophilus* 4461 and 33200 and, *B. longum* 5022 in particular, were capable of growing well in RSM due to their proteolytic activities. When grown individually, *S. thermophilus* showed better growth than *L. delbrueckii* ssp. *bulgaricus* and the probiotics, which grew slowly. The increase in growth translated in corresponding decrease in pH. Among all the organisms, only the extracellular extract of *S. thermophilus* 1275 and the intracellular extracts of *L. acidophilus* 4461 and *L. delbrueckii* ssp. *bulgaricus* 1092 exhibited aminopeptidase activities to all the substrates tested while the dipeptidases of all organisms exhibited broad substrate specificity and could utilise all the substrates studied. All the organisms showed varying capabilities to generate bioactive components that could inhibit ACE and α-glu activities *in vitro*. Among the species of organisms studied, *B. longum* 5022 showed very high proteolytic and ACE-inhibitory activities.

Addition of Versagel® to RSM resulted in improved growth of the selected strains of *S. thermophilus* 1275 and *B. longum* 5022 but inhibited that of *L. casei* 15286, *L. acidophilus* 4461 and *L. delbrueckii* ssp. *bulgaricus* 1368. This was also reflected in the extent of reduction of pH by these organisms. Among the biochemical activities, proteolytic activity of almost all the organisms except *B. longum* 5022, was adversely affected by the presence of Versagel although the ACE-inhibitory and α-glu inhibitory activities were improved. The rate of addition of Versagel mainly influenced the growth, ACE-inhibitory and α-glu inhibitory activities of the selected organisms. Thus, it is appears that fat replacers such as Versagel can influence the growth and related biochemical activities of organisms in fermented milks.

The bifidogenic nature of Raftiline HP® was confirmed based on the significantly improved growth of *B. longum* 5022. Addition of Raftiline HP also improved the growth of *S. thermophilus* 1275. All organisms produced more lactic acid when grown in the presence of 1% Raftiline HP but there was no effect on reduction of pH of the medium with the exception of *B. longum* 5022 and *L. delbrueckii* ssp. *bulgaricus* 1368. Incorporation of Raftiline HP, as well as the level of addition, had a varying influence on the proteolytic capabilities depending on the type of organism while the generation of ACE-inhibitory peptides by *L. casei* 15286, *L. delbrueckii* ssp. *bulgaricus* 1368 and *B. longum* 5022 only was improved. However, there was an improvement in the α-glu inhibitory activities of all the organisms in RSM added with 3% Raftiline HP, except that of *L. delbrueckii* ssp. *bulgaricus* 1368. All organisms showed lower α-glu inhibitory activities in RSM added with

2% Raftiline HP. The level of addition of Raftiline HP showed varying influences on the growth and biochemical activities of the organisms. Thus, it appears that Raftiline HP not only enhanced the growth of *B. longum* 5022 but also improved some of its biochemical activities. These observations can be useful in deciding the level of Raftiline HP required to be added for improved bioactivity of the organisms used in food fermentations.

Incorporation of Versagel as a fat replacer in the preparation of low-fat yogurt decreased the fermentation time during yogurt making. The pH of yogurts containing Versagel was higher than the control while the concentration of lactic and acetic acids was similar in the fresh samples of low-fat yogurt regardless of the presence of Versagel. Although there was an increase in the concentration of lactic acid in the three types of yogurt, there was no change in the level of acetic acid during the storage of the yogurts. The starter cultures, *S. thermophilus* 1275 and *L. delbrueckii* ssp. *bulgaricus* 1368, maintained their viability in all the yogurts throughout the storage period. However, the proteolytic and ACE-inhibitory potential of the starter cultures was suppressed in the presence of Versagel. On the other hand, the addition of Versagel had a positive impact on the physical properties of the low-fat yogurt. The amount of spontaneous whey separation reduced while firmness and pseudoplastic properties of the low-fat yogurts improved in low-fat yogurts containing Versagel. Thus, Versagel can be useful in improving the textural characteristics of low-fat yogurt but has a negative impact on proteolytic and ACE-inhibitory potential.

Incorporation of Raftiline HP appeared to improve the growth of starter organisms which resulted in shorter fermentation time of low-fat yogurt. Raftiline HP supported the growth of *L. delbrueckii* ssp. *bulgaricus* 1368 irrespective of the level of addition but did not help the growth of *S. thermophilus* 1275 as much, particularly at 2% level. However, there were no noteworthy changes in pH during storage nor any increase in concentration of lactic acid in the low fat yogurts containing Raftiline HP compared to control. Improvement in total proteolysis in the yogurts was observed in the presence of Raftiline HP and was highest in yogurt containing 3% Raftiline HP. Consequently, the ACE-inhibitory activity was maximal in yogurt containing 3% Raftiline HP all through the storage of the yogurts, indicating better ACE-inhibitory activity as compared to those containing 2% Raftiline HP and the control yogurt. Incorporation of Raftiline HP did not affect the textural properties of the low-fat yogurts such as whey separation and firmness. The products were more fluid like than the control with distinct pseudoplastic properties and lesser ability to resist deformation upon applied shear.

When the yogurts differing in the strain of *S. thermophilus*, EPS-producing 1275 vs. non-EPS-producing 1342, were compared during storage of the low-fat yogurts, the presence of EPS did not have any influence on changes in pH and lactic acid content although there was a protective effect on the survival of *L. delbrueckii* ssp. *bulgaricus* 1368 and to some extent on that of *S. thermophilus* 1275. The EPS content of yogurt dropped substantially during the first week of storage and remained stable thereafter. There was considerable increase in the extent of proteolysis in EPS containing at d 14 and in yogurt without EPS at d 14 and 21of storage. Exopolysaccharides did not appear to have any influence on the ACE-inhibition activity. However, there was a definite influence of EPS in reducing the firmness, spontaneous whey separation and yield stress of low-fat yogurt whereas there was no influence on the consistency index and flow behaviour index of the yogurts. Yogurts made from EPS-producing starter showed a faster structural recovery after shear and exhibited better viscous properties. Thus, use of EPS producers in low-fat yogurt improved the textural properties of the yogurts without influencing their ACE-inhibition potential.

The presence of EPS-producing strain of *S. thermophilus* 1275 along with inulin did not affect the pH and lactic acid concentration of the low-fat yogurts but exhibited a protective effect on the survival of *L. delbrueckii* ssp. *bulgaricus* 1368 during storage at 4 °C. No change in the EPS content was observed during storage of the inulin containing low-fat yogurts, except for a sharp increase at d 14. The proteolytic activity of yogurt produced with EPS-producing strain of *S. thermophilus* 1275 and inulin was higher than the control at d 1, 21 and 28, indicating a time-dependant effect of EPS. The ACE-inhibitory activity varied with the time of storage in both the types of yogurt, being higher in EPS containing yogurt at d 28. The EPS and inulin containing yogurt showed better α-glu-inhibitory activity as compared to the control. Yogurts made with EPS-producing strain of *S. thermophilus* 1275 and inulin appeared to have a stable and compact structure as indicated by the reduction in appearance of spontaneous whey separation, lower firmness, G′ values and yield stress. The consistency index and hysteresis loop area were also lower in the EPS and inulin containing yogurt.

Further, the presence of EPS-producing strain of *S. thermophilus* 1275 did not affect pH and lactic acid concentration of the inulin containing low-fat probiotic yogurts. However, EPS exhibited a protective effect on the survival of *L. delbrueckii* ssp. *bulgaricus* 1368 during storage at 4°C but not on that of *L. casei* 15286 and *B. longum* 5022 while some improvement in the survival of *L. acidophilus* 4461 was observed. The EPS content increased until d 21 after which a sharp decrease was observed. The proteolytic activity of

yogurt produced with EPS-producing strain of *S. thermophilus* 1275 was higher than the control after d 14. No specific protective effect of EPS was observed regarding the ACE-inhibitory activity. The textural and rheological characteristics of EPS containing yogurt differed in the presence of probiotics. The firmness and storage and loss moduli were similar in control and EPS containing yogurt during the first two weeks of storage and no decrease in the spontaneous whey separation of EPS containing yogurt was observed as compared to the control. Thus, it appears that the influence of EPS on the rheological parameters was observable only after a week of storage. The influence of EPS on the rheological parameters of yogurt differs in the presence of probiotic organisms. This aspect needs further investigation.

The animal feeding trial with SHR indicated that the overall weight gains were lesser in the groups fed yogurt containing diets than skim milk containing diets being in the order RC (90 g) > RPY (85.7g) > RY (78.7g), during the 8 wk feeding period. During feed preparation, the ACE-inhibitory activity decreased when the yogurts were freeze dried but increased in the corresponding pelleted diets. A definite antihypertensive effect was observed in SHR fed the diets containing the yogurts. At the end of the feeding period, taking the systolic and diastolic BP of control group as baseline, the reduction in the group fed yogurt supplemented diet was -9.46 and -9.41 mm Hg while in the group fed probiotic supplemented diet, it was -6.91 and -13.84 mm Hg, respectively. It was concluded that feeding the diets containing yogurts reduced the systolic and diastolic BP of the SHR rats as compared to the control diet and that yogurt supplemented diets had a better ability to maintain the lowered systolic but not diastolic BP than probiotic yogurt supplemented diet. The lipid profile of SHR group fed probiotic yogurt supplemented diet showed a 35% reduction in LDL-cholesterol and 26.2% reduction in total cholesterol while in the group fed yogurt supplemented diet the reductions were 30% and 23.3%, respectively, as compared to those of RC, suggesting a synergistic effect of yogurt starters and probiotics in lowering the LDL-cholesterol levels in SHR. The reduction in AI and ratios of total cholesterol to HDL-cholesterol and LDL-cholesterol to HDL-cholesterol indicated that the hypocholesterolemic effect accrued as a consequence of feeding yogurt and probiotic yogurt supplemented diets to SHR. In summary, our results suggest that supplementation (~ 45%) of skim milk diet of SHR with freeze dried yogurt, with or without probiotics, aided in controlling weight gain, systolic and diastolic BP, serum TG, total cholesterol, and LDL-cholesterol without affecting the serum HDL-cholesterol levels. Therefore, it was concluded that it is possible to manufacture yogurt having multiple health benefits, having both serum lipid improvement

and hypotensive effect on SHR. However, these effects need to be confirmed in human studies.

Some general conclusions from the whole research project were:

a. Additives such as fat replacers affected the biochemical activities of organisms, thereby making it necessary that the effect of additives be considered before including in fermented products. These effects can vary when more than one organism is co-cultured as in yogurt making.
b. Raftiline HP was a better choice of fat replacer than Versagel when considering the therapeutic potential of the low-fat yogurt. However, if only the textural properties were to be considered, Versagel was a better choice of fat replacer in low-fat yogurt.
c. Proteolytic activity cannot be taken as a confirmed indicator of ACE-inhibitory potential of the product.
d. The production of ACE-inhibitory peptides *in situ* and during storage was variable.
e. Co-culturing did not necessarily improve ACE-inhibitory activity in the fermented product.
f. The ACE-inhibitory activity was influenced by the type of cultures used for fermentation and the level of Raftiline HP.
g. Presence of EPS did not show any influence on ACE-inhibitory activity.
h. Although ACE-inhibitory activity *in vitro* was low, the antihypertensive effect was substantial when yogurt containing feeds were fed to spontaneously hypertensive rats implying that either more potent ACE-inhibitory peptides were generated and assimilated during the transit of the feed through the GIT or the antihypertensive effects were affected through mechanisms other than ACE-inhibition.
i. EPS improved the textural properties of low-fat yogurt particularly, spontaneous whey separation along with softer gel. However, the influence of EPS on the textural characteristics of yogurt was modified in the presence of probiotics but not in the presence of Raftiline HP.
j. The quantity of EPS produced in low-fat yogurts increased in the presence of Raftiline HP and probiotics.
k. Co-culturing had a negative impact on the *in vitro* antidiabetic potential of the low-fat yogurt as measured by the extent of α-glucosidase inhibition. The effect was observed only in the presence of inulin.

9.0 Future Research Direction

This research has raised some interesting questions that need to be addressed with further research. These can be classed under three major areas of research.

The ACE-inhibitory activity was the major thrust of this work. Given that products will be stored post-manufacture until consumption, it becomes important that the ACE-inhibition that is claimed in the product at the time of production be maintained until the end of its storage period. We found ACE-inhibition to be a highly variable parameter. Thus, apart from strain selection, the factors that affect the stability of the ACE-inhibitory peptides in the product need to be understood. Also, it would be interesting to identify the changes in the peptide profile that causes these changes. Moreover, we have observed that despite relatively low *in vitro* activity, good antihypertensive effect was exhibited when the product was fed to SHR. We need to find out if there are other mechanisms through which the antihypertensive effect is being exhibited by the product. We also need to confirm these effects by conducting human trials. Also considering that the product showed hypocholesterolemic effect, it would be interesting to see if any of the peptides generated during fermentation were responsible for this effect.

Another area of research is related to EPS. We found that the effect of EPS on yogurt texture differed in the presence of inulin and probiotics. It would be interesting to study the microstructure of the products during fermentation and during storage to see what causes these differences. We also observed that the quantity of EPS produced *in situ* increased in the presence of inulin and probiotics so also their behaviour. Whether these adjuncts bring about a change in the composition of EPS and how it causes changes in textural properties of EPS produced will be another challenging area of research.

We found that the organisms we used showed α-glucosidase inhibitory activity which indicates its potential to control blood sugar levels. However, α-glucosidase inhibitory activity was not exhibited when the organisms were co-cultured during yogurt making but were present when inulin was incorporated in the yogurt. It would be interesting to examine why this sort of variation was observed and what are the components generated during fermentation that showed this activity.

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