INFLUENCE OF PROBIOTIC ORGANISMS ON RELEASE OF BIOACTIVE COMPOUNDS IN YOGHURT AND SOY YOGHURT

A thesis submitted for the degree of Doctor of Philosophy

By

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I dedicate this PhD to my late father, Mr. Bernard Francis Donkor

who believed so much in education

&

to the entire Donkor family

ABSTRACT

Viability and metabolic activities are important characteristics of probiotic microorganisms. They give rise to therapeutic benefits as well as increase physiological activity of cultured products through liberation of a number of bioactive peptides and activation of latent compounds such as conjugated isoflavones. The main focus of this project was to assess the performance of commercial probiotic strains in regard to viability, metabolic activity and ability to produce bioactive compounds.

The viability of *Lactobacillus acidophilus* LAFTI® L10, *Bifidobacterium lactis* LAFTI® B94, and *Lactobacillus paracasei* LAFTI® L26 and their proteolytic activities were assessed in yoghurt at different batches by fermentations terminated pH of 4.45, 4.50, 4.55, and 4.60 in the presence of *L. delbrueckii* ssp. *bulgaricus* Lb1466 and *S. thermophilus* St1342 during 28 days of storage at 4°C. All strains achieved the recommended level of 6.00 log cfu/g of the product with *L. acidophilus* LAFTI® L10 and *L. paracasei* LAFTI® L26 exceeding the number to 8.00 and 7.00 log cfu/g, respectively. Lactobacilli strains showed a good cellular stability maintaining constant concentration throughout storage period regardless of termination pH. On the other hand, the cell counts of *B. lactis* LAFTI® B94 decreased by one log cycle at the end of storage. The presence of probiotic organisms enhanced proteolysis significantly in comparison to the control batch containing *L. delbrueckii* ssp. *bulgaricus* Lb1466 and *S. thermophilus* St1342 only. The proteolytic activity varied due to termination pH, but also appeared to be strain related. The increased proteolysis improved survival of *L. delbrueckii* ssp. *bulgaricus* Lb1466 during storage resulting in further lowering of pH and production of higher levels of organic acids, which might have caused the low cell counts for *Bifidobacterium lactis* LAFTI® B94.

Furthermore, these organisms were cultured also in soymilk to determine the suitability of soy yoghurt as a system for delivering probiotics and other bioactive compounds. Fermentations were terminated at different pH of 4.50, 4.55, and 4.60 and metabolic patterns of cultures (viability, proteolytic activity, organic acids production, ACE inhibitory activity) were investigated during 28 days storage at 4°C. The presence of probiotics enhanced the growth of *L. delbrueckii* ssp. *bulgaricus* Lb1466 and *S. thermophilus* St134 in soy yoghurt in comparison to the control produced by sole yoghurt culture. In general, different termination pH had no effect ($P > 0.05$) on the viability of probiotic organisms which maintained good viability in soy yoghurt during cold storage. Higher levels of essential growth factors in the form of peptides and amino acids in soy

yoghurts may have promoted the growth of *L. acidophilus* LAFTI® L10, *B. lactis* LAFTI® B94 and *L. paracasei* LAFTI® L26. The use of probiotic strains as a part of starter culture in soy yoghurt resulted in a substantial increase in *in vitro* ACE inhibitory activity in comparison to the control produced by yoghurt culture only. This improvement of ACE inhibition in soy yoghurt is partly due to higher proteolytic activity of probiotics.

Based on these experiments, we selected two probiotic organisms (*Lactobacillus acidophilus* LAFTI® L10 and *L. paracasei* LAFTI® L26) and evaluated their growth characteristics in the presence of prebiotics. The growth and metabolism of these probiotic organisms and a regular yoghurt culture (*L. delbrueckii* ssp. *bulgaricus* Lb1466 and *Streptococcus thermophilus* St1342) were studied in yoghurt containing (0.5, 1.0, and 1.5)% (w/v) of high amylose corn starch powder (Hi-maize®) or inulin. Viable cell counts of probiotic organisms, their metabolites and proteolytic activities, and viscosity of the yoghurts were determined during refrigerated storage for 28 d at 4°C. In the presence of inulin, cultures showed better retention of viability (8.0 log cfu/g) in comparison to that of Hi-maize with a reduction by one log cycle. The production of organic acids also appeared to be growth associated and highly influenced by the types and concentrations of prebiotics. Lower concentrations (0.5-1.0%) of hi-maize improved ($P < 0.05$) the production of propionic acid and also increased proteolytic activity of probiotic organisms substantially. A greater release of free amino acids may have sustained better growth of the organisms in yoghurts. Supplementation with either hi-maize or inulin increased the viscosity of probiotic yoghurts significantly ($P < 0.05$).

Since proteolytic activity of microorganisms is important for growth and the production of bioactive compounds in milk and soymilk, the enzymatic (proteolytic) systems of these organisms were studied. Two strains each of *Lactobacillus acidophilus*, *Bifidobacterium sp.*, and *Lactobacillus casei*, and one strain each of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus* were assessed for growth characteristics, proteolytic activity and release of *in vitro* angiotensin-converting enzyme inhibitory peptides in reconstituted skim milk. Single cultures grew well with exception of *Lactobacillus delbrueckii* ssp. *bulgaricus*. Despite slow growth, this culture produced substantial amount of lactic acid, second to *S. thermophilus*. All strains exhibited proteolytic activities with intra- and extracellular specific peptidases including X-prolyl-dipeptidyl aminopeptidase. The latter cleaved proline-containing sequences, which possibly enhanced

liberation of various peptides and likely resulted in improved cell growth. The extent of proteolysis varied among strains and appeared to be time dependant. All the cultures released peptides with *in vitro* ACE-inhibitory activity during growth with *B. longum* Bl 536 and *L. acidophilus* L10 having IC_{50} values of 0.196 and 0.151 mg/mL, respectively.

Again, the metabolic activities of these selected strains (*Lactobacillus acidophilus* (LAFTI® L10 and La4962) *Bifidobacterium* (*lactis* LAFTI® B94 and *longum* Bl536), *L. casei* (LAFTI® L26 and Lc279), *L. delbrueckii* ssp. *bulgaricus* Lb1466 and *S. thermophilus* St1342 were each investigated in soymilk. Strains were initially analyzed for α-galactosidase activity and organic acid production in MRS broth at 37°C. Consequently, soymilk was fermented with each strain and cell growth, production of organic acid, metabolism of oligosaccharides and proteolytic and ACE-inhibitory activities were assessed during 48 h of incubation at 42°C. All strains exhibited variable α-galactosidase activity with *Bifidobacterium lactis* B94 showing the highest activity. The oligosaccharide metabolism depended on α-galactosidase activity. *B. lactis* B94, *S. thermophilus* St1342 and *L. acidophilus* La 4962 reduced raffinose substantially by 77.4, 64.5 and 55.9%, respectively. All strains reached the desired therapeutic level of 10^8 cfu/mL in soymilk after 48 h at 42°C. The hydrolysis of protein in soymilk likely depended on strain ($P < 0.0001$) and time ($P < 0.0001$). The strains also released bioactive peptides with ACE-inhibitory activities between 17 and 43%.

The study determined β-glucosidase activity of commercial probiotic organisms for hydrolysis of isoflavone to aglycones in fermenting soymilk. Soymilk made with soy protein isolate (SPI) was fermented with *Lactobacillus acidophilus* LAFTI® L10, *Bifidobacterium lactis* LAFTI® B94 and *Lactobacillus casei* LAFTI® L26 at 37 °C for 48 h and the fermented soymilk was stored for 28 d at 4 °C. β-Glucosidase activity of organisms was determined using ρ-nitrophenyl β-D-glucopyranoside as a substrate and the hydrolysis of isoflavone glycosides to aglycones by these organisms was carried out. The highest level of growth occurred at 12 h for *L. casei* L26, 24 h for *B. lactis* B94, and 36 h for *L. acidophilus* L10, during fermentation in soymilk. Survival after storage at 4 °C for 28 d was 20, 15 and 11% greater ($P < 0.05$) than initial cell counts respectively. All the bacteria produced β-glucosidase which hydrolyzed isoflavone β-glycosides to isoflavone aglycones. The decrease in the concentration of β-glycosides and the increase in the concentration of aglycones were significant ($P < 0.05$) in the fermented soymilk. Increased isoflavone

aglycone content in fermented soymilk is likely to improve the biological functionality of soymilk.

An attempt was made to analyse the peptide fractions from different yoghurt batches for their *in vitro* ACE inhibitory (ACE-I) activity. Inhibition of angiotensin I-converting enzyme (ACE) activity results in an overall antihypertensive effect. Yoghurts were prepared either using a sole yoghurt culture including *L. delbrueckii* ssp. *bulgaricus* Lb1466 and *S. thermophilus* St1342 or *L. acidophilus* L10, *L. casei* L26 and *B. lactis* B94 in addition to yoghurt culture. The ACE-I activity was determined at weekly intervals during 28 days of cold storage. Peptide fractions showing high ACE-I activity were further purified using multiple-steps of RP-HPLC. All probiotic yoghurts showed an appreciable ACE-I activity during initial stages of storage compared to the control yoghurt with a significant ($P < 0.05$) decrease afterwards. The ACE-I activity ranged from IC_{50} of 103.30 to 27.79 μg /mL with the greatest ACE inhibition achieved during first and third week of storage. The *in vitro* ACE-I activity could be related to the peptide liberation via degradation of caseins. In total 8 ACE-I peptides were characterized originating from α_{s2}-CN (1), κ-CN (2) and β-CN of which two well-known ACE-inhibiting peptides, namely Val-Pro-Pro (VPP) and Ile-Pro-Pro (IPP), were identified. These peptides are already used in commercial products.

In the final study, the applicability of probiotic organisms in the production of soy yoghurt and the chemical compositions, rheological and sensory properties of the product were examined during 28 day storage at 4°C. Soymilk supplemented with 2% (w/v) inulin or 1% (w/v) each of raffinose and glucose was used as a base for soy yoghurt manufacture. Viability of probiotic organisms and their metabolic activity measured as production of organic acids, aldehyde content responsible for beany flavour, as well as rheological and sensory properties of soy yoghurt were examined. Inulin or raffinose/glucose supplementation in soymilk increased the bacterial population by one log cycle and the amount of lactic acid. Probiotic bacteria metabolized more aldehyde than yoghurt culture and substantially reduced the beaniness in soy yoghurt as determined by sensory evaluation. The probiotic soy yoghurts showed more viscous and pseudo-plastic properties than the control soy yoghurts but the sensory evaluation results showed preference for the control soy yoghurts which were slightly less viscous. Control soy yoghurt provided better mouthfeel than probiotic soy yoghurts.

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CERTIFICATE

This is to certify that the thesis entitled "INFLUENCE OF PROBIOTIC ORGANISMS ON RELEASE OF BIOACTIVE COMPOUNDS IN YOGHURT AND SOY YOGHURT" submitted by Osaana N. Donkor in partial fulfillment of the requirement for the award of the Doctor of Philosophy in Food Technology at Victoria University is a record of bonafide research work carried out by him 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 Osaana Nti Donkor, declare that the PhD thesis entitled *Influence of Probiotic Organisms on release of Bioactive Compounds in Yoghurt and Soy Yoghurt* 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".

Osaana N. Donkor Date:

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Refereed Research Papers

Donkor, O. N., Henriksson, A., Vasiljevic, T., & Shah, N. P. (2005). Probiotic strains as starter cultures improve angiotensin-converting enzyme inhibitory activity in soy yoghurt. *Journal of Food Science*, *70*, M375-M381.

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Chaired Oral Presentations

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Table of Contents

Chapter **Page**

List of Tables

Table **Page**

List of Figures

List of Abbreviations

μ = micro

κ-CN = kappa-casein

μg = microgram

μL = microlitre

α-La = alpha-lactalbumin

β-Lg = beta-lactoglobulin

μm = micrometre

ANOVA = analysis of variance

Bb = *Bifidobacterium*

BSA = bovine serum albumin

CE = Capillary electrophoresis

cfu = colony forming unit

CN = casein

ECD = electrochemical detector

FAO = Food and Agricultural Organization of the United Nations

FID = Flame ionization detector

g = gram

GC = Gas chromatography

h = hour

H_2SO_4 = sulphuric acid

HCl = hydrochloric acid

He = helium

HS = Head space

Ig = immunoglobulin

L = litre

La = *Lactobacillus acidophilus*

Lb = *Lactobacillus delbrueckii* ssp. *bulgaricus*

M17 agar = agar for enumeration of *Streptococcus thermophilus*

min = minute

mL = millilitre

mm = millimetre

mM = millimolar

MRS = de Man Rogosa and Sharpe

mV = millivolts

MW = molecular weight

nA = nanoamps

ng = nanogram

NNLP = nalidixic acid, neomycin sulphate, lithium chloride and paramomycin sulphate

^{o}C = degree Celsius

OPA = o-phthaldialdehyde

Pa = Pascal

pH = hydrogen ion concentration

RCA = reinforced clostridia agar

RP-HPLC = reversed-phase high performance liquid chromatography

rpm = revolution per minute

RSM = reconstituted skim milk

s = second

SMP = skim milk powder

sp. = species

ssp. = subspecies

St = *Streptococcus thermophilus*

TCA = trichloroacetic acid

TFA = trifluoroacetic acid

UV = ultra violet

V = volts

v/v = volume per volume

w/w = weight per weight

WHO = World Health Organization

x g = times gravitational force

1.0 Introduction

Probiotic organisms are 'live micro-organisms which, when consumed in adequate amounts, confer a health benefit on the host' (FAO/WHO, 2002). To observe a positive health effect of their consumption, a minimum level of live microorganisms is required; this level, depending on the strains used and the required health effect, is usually between 10^8 and 10^{11} cfu/d (Vanderhoof and Young, 1998). Therefore, assuming a daily consumption of fermented dairy products of 100 g, they should contain between 10^6 cfu/g to 10^9 cfu/g of these live bacteria at the time of consumption. While this is the case for a limited number of strains, the precise mechanism by which probiotic organisms exert a health effect *in vivo* is not clearly understood. One aspect that is clear, however, is that some strains produce certain health-promoting metabolites – including proteins and fatty acids – which are desirable from a nutritional and/or physiological perspective. However, it should be emphasised that the ingestion of probiotic fermented foods opens up the possibility that these health-promoting metabolites may also be produced *in vivo* (Tamime, 2005).

Epidemiological studies suggest that consumption of fermented milk and the dietary intake of dairy proteins are inversely related to the risk of hypertension, resulting from a blood pressure lowering effect of released bioactive peptides (Jaubiainen and Korpela, 2003). Bioactive peptides are defined as specific protein fragments that have a positive impact on body functions or conditions and may ultimately influence health (Kitts and Weiler, 2003). Upon oral administration, bioactive peptides may affect the major body systems, namely, the cardiovascular, digestive, immune, and nervous system, depending on their amino acid sequence. These peptides are formed through the enzymatic breakdown of milk proteins by either digestive enzymes in the gastrointestinal tract (GIT) or extracellular proteinases released by lactobacilli during their growth in milk. The tripeptides, isoleucyl–prolyl–proline (Ile-Pro-Pro) and valyl-prolyl-proline (Val-Pro-Pro), have been identified as antihypertensive agents, which inhibit the action of angiotensin converting enzyme (ACE) (Jaubiainen and Korpela, 2003). Several investigations have been carried out on the fermentation of milk by *L. helveticus* and *Saccharomyces cerevisiae* (Pihlanto and Korhonen, 2003), there is little information on the release of bioactive peptides by *L. acidophilus*, *Bifidobacterium* sp., and *L. casei* during growth in bovine yoghurt.

Soymilk based yoghurts offer a considerable appeal for a growing segment of consumers with certain dietary and health concerns. It has several nutritional advantages such as reduced

level of cholesterol and saturated fat as well as the absence of lactose (Favaro Trindade *et al*., 2001). Soybean milk, which serves as a base for a variety of beverages, also contains raffinose, stachyose, pentanal, n-hexanal and phytoestrogens. Some strains of *Bifidobacterium* are able to reduce the concentration of raffinose and stachyose, eliminating the potential cause of flatulence, and also decrease the levels of pentanal and n-hexanal responsible for the beany flavour (Desai *et al*., 2002; Tsangalis and Shah, 2004).

In addition, soymilk contains phytoestrogens, which are plant-derived phenolic compounds with a structural homology to human estrogens. These compounds consist of the di-phenolic, isomeric group known as isoflavones. Raw soybeans typically contain 2-4 mg of isoflavones/g, whereas the isoflavone content of soyfoods, ranges from about 1 to 3 mg/g on a dry weight basis. Some isoflavones are lost during the processing of soybeans, for example, when drying and milling of soybeans to soy flour or in preparation of soy protein isolate. The loss of isoflaveones is particularly marked in the case of certain brands of soy protein concentrate. The conventional method for making this product includes an aqueous alcohol wash of soy flakes. This is done to remove soluble carbohydrates, but results in the loss of isoflavones along with many other phytochemicals.

One serving of a traditional soyfood such as one cup of soymilk or ½ cup of tofu contains about 30 mg of isoflavones, which is quite high considering that the dietary intake of flavonoids of many populations is less than this amount (Hertog *et al*., 1995). More importantly, there are clinical data indicating the amount of isoflavones in about 1-2 servings of soyfoods that exert marked physiological effects (Messina *et al*., 1994). The intestinal microflora can convert daidzein into several different products including the isoflavone equol, dihydrodaidzein, and O-desmethylangolensin (ODMA), although because of differences in intestinal microflora, not all subjects fed soymilk produced equol (Kelly *et al*., 1995). In soybeans, there is typically more genistein than daidzein (almost a 2:1 ratio) (Wang and Murphy, 1994), but the ratio in individual soy products can vary greatly. For example, genistein to daizein ratio in miso, tempeh, soy flour, and soymilk has been reported to be 1.43, 1.82, 1.30, and 1.21 respectively, and in two different brands of tofu, the ratios were 2.04 and 1.35 (Coward *et al*., 1993).

Bovine yoghurt is a fermented milk product made with a mixed starter culture consisting of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus* (Shah, 2003; Tamime and Deeth, 1980). Bovine yoghurts available on market can be classified according to

the physical properties of gels (set, stirred and drinking), the application of probiotic bacteria or prebiotics (probiotic- or prebiotic bovine yoghurt), fat content (full-fat, reduced fat and low-fat) and the addition of flavour (such as sweeteners, fruits or flavouring). Furthermore, there are other variations of products including frozen-, concentrated-, dried-, low-calorie-, low-lactose- and pasteurized bovine yoghurt (Tamime and Deeth, 1980; Shah, 2003). Soy yoghurt is a fermented soymilk made with a mixed starter culture consisting of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*. The types of soy products available on market include soy-based cheeses, tofu, soy yoghurt, soy ice cream, soy sauce and soy vegetable burgers. Also soybean flour, soybean concentrates and isolated soy proteins are some of ingredients used in the food industry (Wiseman, 1997; Liu, 1997). The next chapter which deals with the literature review will focus on set bovine yoghurt and soy yoghurt only, as these products have been used in this study.

The main goal of this project was to study the viability- and influence of selected probiotic organisms on the formation of the functional compounds (bioactive peptides, and bioactive isoflavones) in bovine yoghurt and soy yoghurt with improved sensory attributes. The specific objectives were:

1. to examine survival of selected probiotic organisms during the manufacture and storage of bovine yoghurt and soy yoghurt,
2. to assess the effect of inoculum size, strike pH and use of various combinations of cultures to prevent post-acidification in bovine yoghurt and improve survival of probiotic bacteria during storage at 4^{o}C,
3. to investigate the effects of selected prebiotics on growth, activity and viability of probiotic bacteria in bovine yoghurt and soy yoghurt,
4. to examine the role of probiotic organisms in metabolism of *n*-hexanal, -pentanal and oligosaccharides in soy yoghurt,
5. to evaluate the extent of proteolysis and release of bioactive peptides by selected probiotic organisms during bovine yoghurt and soy yoghurt production,
6. to assess the levels of isoflavone glucosides and bioactive aglycones during fermentation of soymilk, and
7. to examine the influence of probiotic organisms on organic acids production and their impact on sensory and rheological characteristics of soy yoghurt.

Chapter 2.0 of this thesis contains a thorough literature review of the current scientific knowledge on the proposed subject. Chapter 3.0 focuses on the effects of acidification on the activity of probiotics in bovine yoghurt during cold storage. The viability of *Lactobacillus acidophilus* LAFTI® L10, *Bifidobacterium lactis* LAFTI® B94, and *Lactobacillus paracasei* LAFTI® L26 and their proteolytic activities were assessed in bovine yoghurt at different termination pH of 4.45, 4.50, 4.55, and 4.60 in the presence of yoghurt culture during 28 days of storage at 4°C. Chapter 4.0 focuses on the role of probiotic organisms in improving angiotensin-converting enzyme inhibitory activity in soy yoghurt. Suitability of soy yoghurt as a system for delivering probiotics and other bioactive compounds was assessed by fermenting soy milk using starter culture containing *L. delbrueckii* ssp. *bulgaricus* Lb1466, *S. thermophilus* St1342 and probiotic organisms (*Lactobacillus acidophilus* LAFTI® L10, *Bifidobacterium lactis* LAFTI® B94, and *Lactobacillus paracasei* LAFTI® L26). Fermentations were terminated at different pH of 4.50, 4.55, and 4.60 and metabolic patterns of cultures (viability, proteolytic activity, organic acids production, ACE inhibitory activity) were investigated during 28 days storage at 4°C. Chapter 5.0 reports on the survival and activity of selected probiotic organisms in set-type bovine yoghurt during cold storage. The growth and metabolism of two probiotic organisms (*Lactobacillus acidophilus* LAFTI® L10 and *L. paracasei* LAFTI® L26) and starter culture (*L. delbrueckii* ssp. *bulgaricus* Lb1466 and *Streptococcus thermophilus* St1342) used for making yoghurt were studied in bovine yoghurt containing 0.5, 1.0, and 1.5% (w/v) of high amylose corn starch powder (Hi-maize®) or inulin. Chapter 6.0 deals with proteolytic activity of dairy lactic acid bacteria and probiotics as determinant of viability and *in vitro* angiotensin-converting enzyme inhibitory activity in fermented milk. Two strains each of *Lactobacillus acidophilus*, *Bifidobacterium* sp., and *Lactobacillus casei*, and one strain each of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus* were assessed for growth characteristics, proteolytic activity and release of *in vitro* angiotensin-converting enzyme inhibitory peptides in reconstituted skim milk. Chapter 7.0 focuses on α-galactosidase and proteolytic activities of selected probiotic and dairy cultures in fermented soymilk. The metabolic activity of *Lactobacillus acidophilus* (LAFTI® L10 and La4962) *Bifidobacterium* (*lactis* LAFTI® B94 and *longum* Bl536), *L. casei* (LAFTI® L26 and Lc279), *L. delbrueckii* ssp. *bulgaricus* Lb1466 and *S. thermophilus* St1342 was assessed in soymilk. Strains were initially

analyzed for α-galactosidase activity and organic acid production in MRS broth at 37°C. Consequently, soymilk was fermented with each strain and cell growth, production of organic acid, metabolism of oligosaccharides and proteolytic and ACE-inhibitory activities were assessed during 48 h of incubation at 42^{o}C. Chapter 8.0 investigates ACE-inhibitory activity (ACE-I) of probiotic yoghurt. The ACE-I activity of peptide fractions from different bovine yoghurt batches was assessed. Bovine yoghurts were prepared using either a sole yoghurt culture including *L. delbrueckii* ssp. *bulgaricus* Lb1466 and *S. thermophilus* St1342 or *L. acidophilus* L10, *L. casei* L26 and *B. lactis* B94 and *L. delbrueckii* ssp. *bulgaricus* Lb1466 and *S. thermophilus* St1342. Chapter 9.0 deals with organic acid production and hydrolysis of isoflavone phytoestrogens by *L. acidophilus*, *B. lactis* and *L. casei*. Chapter 10.0 evaluates sensory- and rheological properties of set-type soy yoghurt. The overall conclusions and future research directions are included in Chapter 11.0, and all references are listed in Chapter 12.0.

2.0 Literature Review

2.1 Probiotic foods

Incorporation of probiotic organisms such as *Lactobacillus acidophilus, Bifidobacterium* sp., and *L. casei* in fermented products provides a potential to improve the quality of the product and the health status of consumers. Dairy and soy foods may serve as the ideal system for delivery of probiotic bacteria to the human gastrointestinal tract (GIT) due to provision of a favorable environment that promotes the growth and enhances the viability of these microorganisms (Lourens-Hattingh and Viljoen, 2001). Probiotic foods are defined as 'foods that contain live microorganisms, which upon digestion, actively enhance the health of consumers by improving the balance of microflora in the gut' (Fooks *et al*., 1999). A growing public awareness of diet related health issues and mounting evidence regarding health benefits of probiotics have increased consumers demand for probiotic foods.

Numerous health benefits of probiotic organisms have been suggested including reduction of blood cholesterol, improvement of immunity, alleviation of symptoms of lactose intolerance, treatment of diarrhea, anticarcinogenic and antihypertensive properties and biotransformation of isoflavone phytoestrogen to improve hormonal balance in post-menopausal women (Fuller, 1992; Nagata *et al*., 1998; Shah, 2000a; Lourens-Hattingh and Viljoen, 2001; Setchell *et al*., 2003). Probiotic organisms are classified as lactic acid bacteria (LAB) and are important bacteria for food fermentations. They play crucial roles in the fermentation of milk, soy and meat products, and vegetables such as cabbage.

Biochemically, fermentation is the metabolic process in which carbohydrates, proteins, and related compounds are partially oxidized with the release of energy in the absence of any external electron acceptors. The final electron acceptors are organic compounds produced directly from the breakdown of the carbohydrates. Consequently, incomplete oxidation of the parent compound occurs, and only a small amount of energy is released during the process. The product of fermentation consist of some organic compounds that are more reduced than others (Jay, 2000). LAB also contribute to other fermentation process such as the production of wine. The importance of LAB for food fermentation has stimulated research on these bacteria (Konings, 2002).

2.2 Properties of lactic acid bacteria

LAB are described as Gram-positive microorganisms, devoid of cytochromes and prefer anaerobic conditions but are aerotolerant, fastidious, acid-tolerant, and strictly fermentative. These organisms produce lactic acid as the major end product during the

fermentation of carbohydrates (Axelsson, 1998). The most important genera are *Lactobacillus*, *Lactococcus*, *Enterococococcus*, *Streptococcus*, *Pediococcus*, *Leuconostoc*, and *Bifidobacterium*. Based on guanine + cytosine content, Gram-positive bacteria are divided into two major phylogenetic groups. *Bifidobacterium* exhibits relatively higher guanine + cytosine content in the DNA (>55 mol %) and belongs to the *Actinomycetes* branch. Other genera which form part of the *Clostridium* branch have a lower guanine + cytosine content (<55 mol% in the DNA) (Salminen and Wright, 1998). However, *Bifidobacterium* shares certain physiological and biochemical properties with LAB and some common ecological niches such as the gastrointestinal tract (GIT). Therefore, for practical and traditional reasons, *Bifidobacterium* is considered a part of the LAB group.

Members of the LAB are usually subdivided into two distinct groups based on their carbohydrate metabolism. The homofermentative group consisting of *Lactococcus*, *Pediococcus*, *Enterococcus*, *Streptococcus* and some lactobacilli utilize the glycolytic pathway to transform a carbon source mainly into lactic acid (Axelsson, 1998). As opposed to homofermentors, heterofermentative bacteria produce equimolar amounts of lactate, CO_2, ethanol or acetate from glucose using phosphoketolase pathway. Members of this group include *Leuconostoc*, *Weissella* and some lactobacilli. The species belonging to *Enterococcus* genus are frequently found in traditional fermentations and may be included as a component of some mixed starters. However, their deliberate utilization in dairy fermentations still remains controversial, especially since some of the species have now been recognized as opportunistic human pathogens associated with hospital-acquired- and urinary tract infections (Franz *et al*., 1999).

2.2.1 *Lactobacillus*

L. acidophilus contains mainly obligately homofermenters with lactic acid as the major end-product. However, a few are facultative heterofermenters. They occur naturally in the GIT of humans and animals, in the human mouth and vagina, and in some traditional fermented dairy products, such as kefir. They are either microaerophilic, aerotolerant or anaerobic and strictly fermentative with the guanine + cytosine content of their DNA usually between 32 and 53 mol% (Salminen and Wright 1998).

L. acidophilus is a Gram-positive rod with rounded ends that occurs as single cells, as well as in pairs or in short chains. The typical size is 0.6-0.9 µm in width and 1.5-6.0 µm in length. It is a non-motile and non-spore forming organism. Due to their microaerophilic nature, the surface growth on solid media is generally enhanced by anaerobic condition or

reduced oxygen pressure. The organisms require carbohydrates as energy and carbon source as well as nucleotides, amino acids and vitamins. *L. acidophilus* utilizes sucrose more effectively than lactose. The medium supplementation with different micronutrients, such as manganese, oleic acid and esters especially Tween 80, is essential for the growth of most strains. The optimum growth temperature of *L. acidophilus* is between 35-40ºC, although the growth occurs at as high as 45ºC. The acid tolerance varies from 0.3 to 1.9% titratable acidity, with an optimum growth at pH 5.5-6.0 (Shah, 2000b). Due to low content of available peptides and amino acids in milk or soymilk, *L. acidophilus* tends to grow slowly in these media. Furthermore, most strains of *L. acidophilus* do not thrive well in fermented milk due to low pH.

For the cultivation and isolation of *L. acidophilus* strains from pure cultures, MRS agar can be employed as a non-selective medium. However, problems may arise if the isolation and enumeration of *L. acidophilus* is required from a mixed population of different genera of microorganisms. In such case, a selective medium must be employed. For example, MRS medium supplemented with bile or that with pH adjusted to 5.2 would assist growth of *L. acidophilus*. When cultivation and isolation of *L. acidophilus* is required in the presence of *L. casei* and *L. rhamnosus,* selective media for the enumeration of these cultures are used. In this case, a basal agar (BA; 10 g trypton, 10 g Lablemco powder, 5 g yeast extract, 1 g Tween 80, 2.6 g K_2HPO_4, 5 g sodium acetate, 2 g tri-ammonium citrate, 2 g $MgSO_4 \cdot 7H_2O$, 0.05 g $MnSO_4 \cdot 4H_2O$, 12 g bacteriological agar), sorbitol agar, BA-mannitol agar or BA-esculin agar can be used for selective enumeration (Tharmaraj and Shah, 2003).

2.2.2 *Bifidobacterium*

Bifidobacterium is generally characterized as Gram-positive, non-spore forming, non-motile and catalase-negative anaerobes with a special metabolic pathway, which allows it to produce acetic acid in addition to lactic acid in the molar ratio of 3:2. They are fastidious organisms and have special nutritional requirements, thus these bacteria are often difficult to isolate and grow in the laboratory (Shah, 2000b). *Bifidobacterium* species found in humans are *B. adolescentis*, *B. angulatum*, *B. bifidum*, *B. breve*, *B. catenulatum*, *B. dentium*, *B. infantis*, *B. longum*, and *B. pseudocatenulatum*. *B. breve*, *B. infantis*, and *B. longum* are found in human infants. *B. adolescentis*, and *B. longum* are found in human adults (Shah and Lankaputhra, 2002).

Bifidobacterium is a saccharolytic organism and produces acetic acid and lactic acid without generation of CO_2. They are capable of utilizing simple carbohydrates, including

glucose, galactose, fructose and lactose, as well as complex carbohydrates. Fructose-6-phosphate phosphoketolase is the characteristic enzyme, and is the most direct and reliable test for assigning an organism to the genus *Bifidobacterium*. The optimum growth pH is between 6.0 and 7.0, with no growth occurring at pH 4.5-5.0 or below, or above pH 8.0. The strains of this species have the mesophilic optimum growth temperature in the range of 37-41ºC, with the maximum at 43-45ºC and minimum between 25 and 28ºC.

While MRS agar can be used as a non-selective medium for isolation of *Bifidobacterium* sp., MRS-NNLP (nalidixic acid, neomycin sulfate, lithium chloride, and paramomycin sulfate) agar is used as a selective medium for enumeration of *Bifidobacterium* in the presence of *L. delbrueckii* ssp. *bulgaricus*, *S. thermophilus* and *L. acidophilus* (Laroia and Martin, 1991). L-cysteine (0.05%) is an essential nutrient in a medium for the growth of *Bifidobacterium*. When L-cysteine is not present in the media, *Bifidobacterium* either does not grow or forms pin-point colonies. Due to their anaerobic nature, incubation conditions are strictly anaerobic at 37°C for 72 h (Tharmaraj and Shah, 2003).

2.3 Metabolic systems of probiotic organisms

2.3.1 Sugar metabolism

The essential feature of LAB metabolism is carbohydrate fermentation coupled with substrate level phosphorylation. The generated ATP is subsequently used for biosynthetic purposes. LAB exhibit an enormous capacity to degrade different carbohydrates and related compounds. In general, the predominant end-product is lactic acid (>50% of sugar carbon). It is evident, however, that LAB adapt to various conditions and change their metabolism accordingly. This may lead to significantly different end-product patterns (Salminen and Wright, 1998).

2.3.1.1 Fermentation of hexose

The two major pathways for hexose (e.g., glucose) fermentation utilized by LAB are shown in Figure 2.1. Glycolysis or Embden-Meyerhof-Parnas (EMP) pathway, used by all LAB except leuconostocs, group III lactobacilli, oenococci, and weissellas, is characterized by the formation of fructose-1,6-diphosphate (FDP), which is split by a FDP aldolase into dihydroxyacetone-phosphate (DHAP) and glyceraldehyde-3-phosphate (GAP). GAP (and DHAP via GAP) is then converted to pyruvate in a metabolic sequence including substrate level phosphorylation at two sites. Under normal conditions, i.e., excess sugar and limited

access to oxygen, pyruvate is reduced to lactic acid by an NAD^+-dependent lactate dehydrogenase (nLDH), thereby reoxidizing the NADH formed during the earlier glycolytic steps. A redox balance is thus obtained, lactic acid is virtually the only end-product, and the metabolic process is referred to as a homolactic fermentation (Figure 2.1) (Benthin *et al*., 1994; Salminen and Wright, 1998).

The 6-phosphogluconate/phosphoketolase (6-PG/PK) is the other main fermentation pathway. It is characterized by initial dehydrogenation steps with the formation of 6-phosphogluconate, followed by decarboxylation. The remaining pentose-5-phosphate is split by phosphoketolase into GAP and acetyl phosphate. GAP is metabolized in the same way as for the glycolytic pathway, resulting in lactic acid formation. When no additional electron acceptor is available, acetyl phosphate is reduced to ethanol via acetyl CoA and acetaldehyde. Since this metabolism leads to large amounts of other end-products (CO_2, ethanol) in addition to lactic acid, it is referred to as a heterolactic fermentation. However, it should be noted that glycolysis may lead to a heterolactic fermentation under certain conditions and that some LAB, regarded as homofermentative, use the 6-PG/PK pathway when metabolizing certain substrates. In theory, homolactic fermentation of glucose results in 2 moles of lactic acid and a net gain of 2 ATP per mole glucose utilized. Heterolactic fermentation of glucose through the 6-PG/PK pathway gives 1 mole each of lactic acid, ethanol, and CO_2 and 1 ATP/glucose (Kandler, 1983; Salminen and Wright, 1998).

Mannose, galactose, and fructose are other forms of hexose fermented by many LAB. The sugars enter the major pathways at the level of glucose-6-phosphate or fructose-6-phoshpate after isomerization and/or phosphorylation. However, galactose is metabolismed by LAB using a phoshpotransferase system (PTS) for uptake of the sugar, e.g., *Lc. lactis*, *E. faecalis*, and *L. casei*. The galactose-6-phosphate formed by the PTS is metabolized through the tagatose-6-phosphate pathway (Figure 2.1) (Bissett and Anderson, 1974; Benthin *et al*., 1994). Tagatose is a stereoisomer of fructose, and requires separate enzymes to metabolize the tagatose derivatives. The tagatose enters glycolysis at the level of GAP. Many strains in this group also have the capacity to transport galactose with a permease and convert it to glucose-6-phosphate via the Leloir pathway. This pathway is also used by galactose-fermenting LAB that transport galactose with a permease and lack a galactose PTS (Fox *et al*., 1990; Salminen and Wright, 1998).

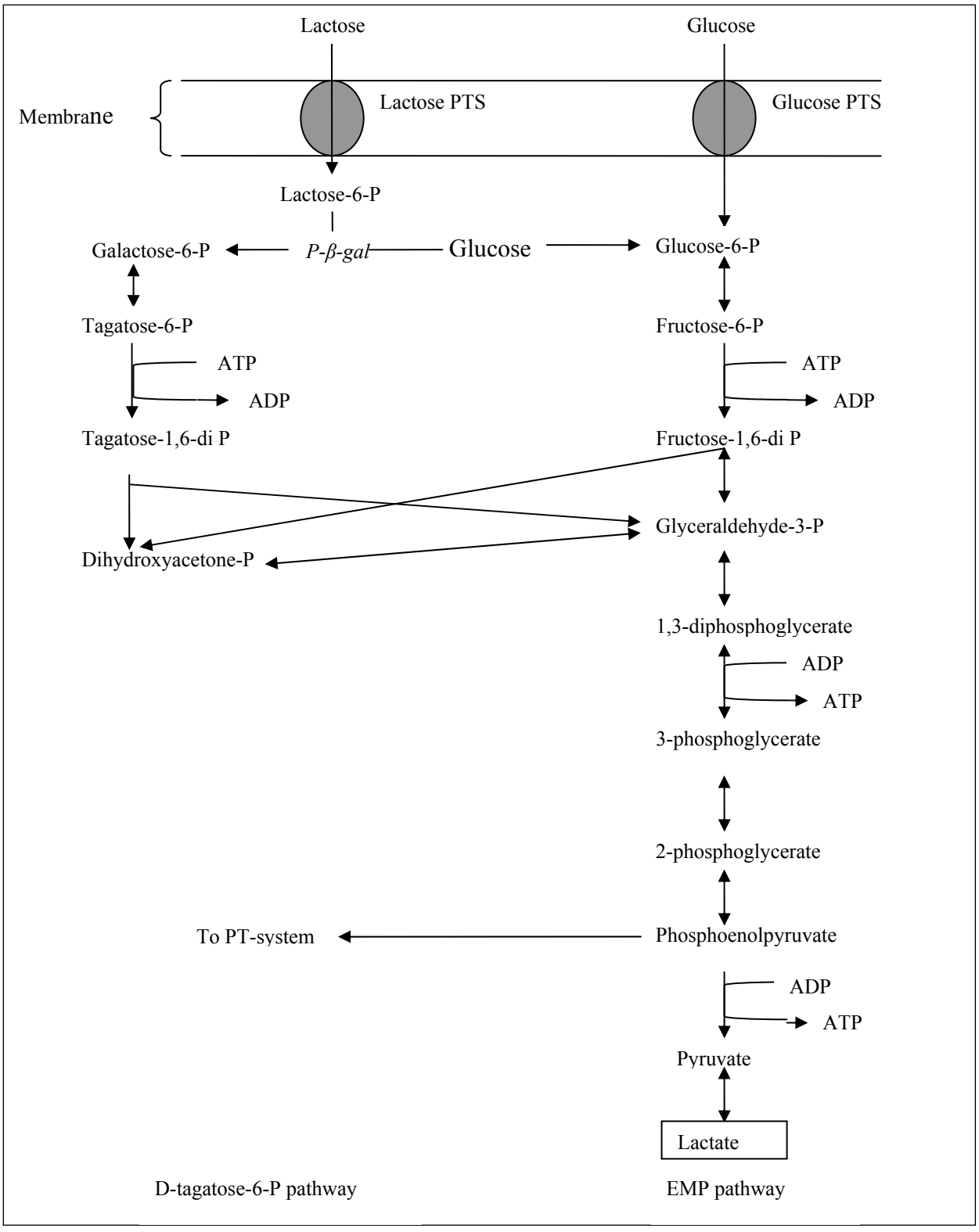

Figure 2.1 Fermentation pathways for lactose and glucose in LAB. Tagatose-6-phosphate pathway and EMP-glycolytic pathway (Benthin *et al*., 1994; Salminen and Wright, 1998).

2.3.1.2 Fermentation of disaccharides

Disaccharides enter the cell either as free sugars or sugar phosphates depending on the mode of transport. Free disaccharides are split by specific hydrolyses to monosaccharides, e.g. lactose to galactose and glucose (Figure 2.2) which then enter the major pathways described above. However, when phosphotransferase systems (PTS) for uptake of sugar are involved, specific phosphohydrolases cleave disaccharide phosphates into monosaccharides and monosaccharide phosphates.

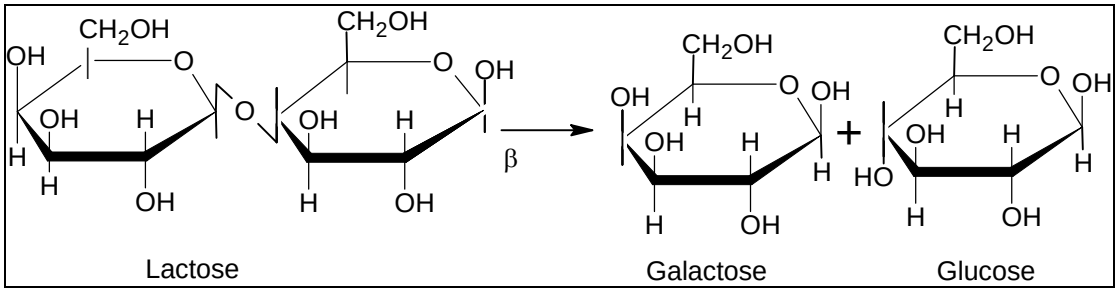

Figure 2.2 Hydrolysis of lactose

2.3.1.2.1 Lactose metabolism

By far the most studied disaccharide metabolism in LAB is lactose fermentation. Most strains used as dairy starters, may contain a lactose PTS (Lac PTS). A lactose PTS from *L. casei* is well characterized (Chassy and Alpert, 1989). In these strains, lactose enters the cytoplasm as lactose phosphate, which is cleaved by phospho-β-D-galactosidase (P-β-gal) to yield glucose and galactose-6-phosphate. Glucose is phosphorylated by glucokinase and metabolized through the glycolytic pathway, whereas galactose-6-phosphate is metabolized through the tagatose-6-phosphate pathway (Figure 2.1). The enzyme system of the lactose PTS and P-β-gal is generally inducible and repressed by glucose. An equally common way to metabolize lactose among LAB is by means of a lactose permease system and subsequent cleavage by β-D-galactosidase (β-gal) to yield glucose and galactose, which may then enter the major pathways (Salminen and Wright, 1998). Some of the "thermophilic" LAB, e.g., *S. thermophilus*, *Lb. delbruckii* ssp. *bulgaricus*, *Lb. delbruckii* ssp. *lactis* and *L. acidophilus* only metabolize the glucose moiety after lactose import and cleavage by β-gal, whereas galactose is excreted into the medium (Figure 2.3) (Hutkins and Morris, 1987; Benthin *et al*., 1994). In *S. thermophilus*, lactose metabolism depends on the lactose transporter protein and low galactokinase activity (Hickey *et al*., 1986; Poolman, 1993).

Expulsion of galactose may be a detoxification process which protects the cell against an inhibitory intracellular concentration of Gal-6P or galactose. Detoxification by dephosphorylation and expulsion of nonmetabolizable phosphorylated sugar analogs, e.g., 2-deoxy-D-glucose and methyl-3-D-thiogalactopyranoside, in lactococci is well known (Thompson & Saier, 1981). Benthin *et al*. (1994) showed that *Lactococcus lactis* ssp. *cremoris* excreted dephosphorylated metabolizable sugar phosphate into the medium. The expulsion of a nonmetabolizable free sugar (the galactose moiety of lactose) is also well known from lactococci unable to metabolize galactose (Benthin *et al*., 1994).

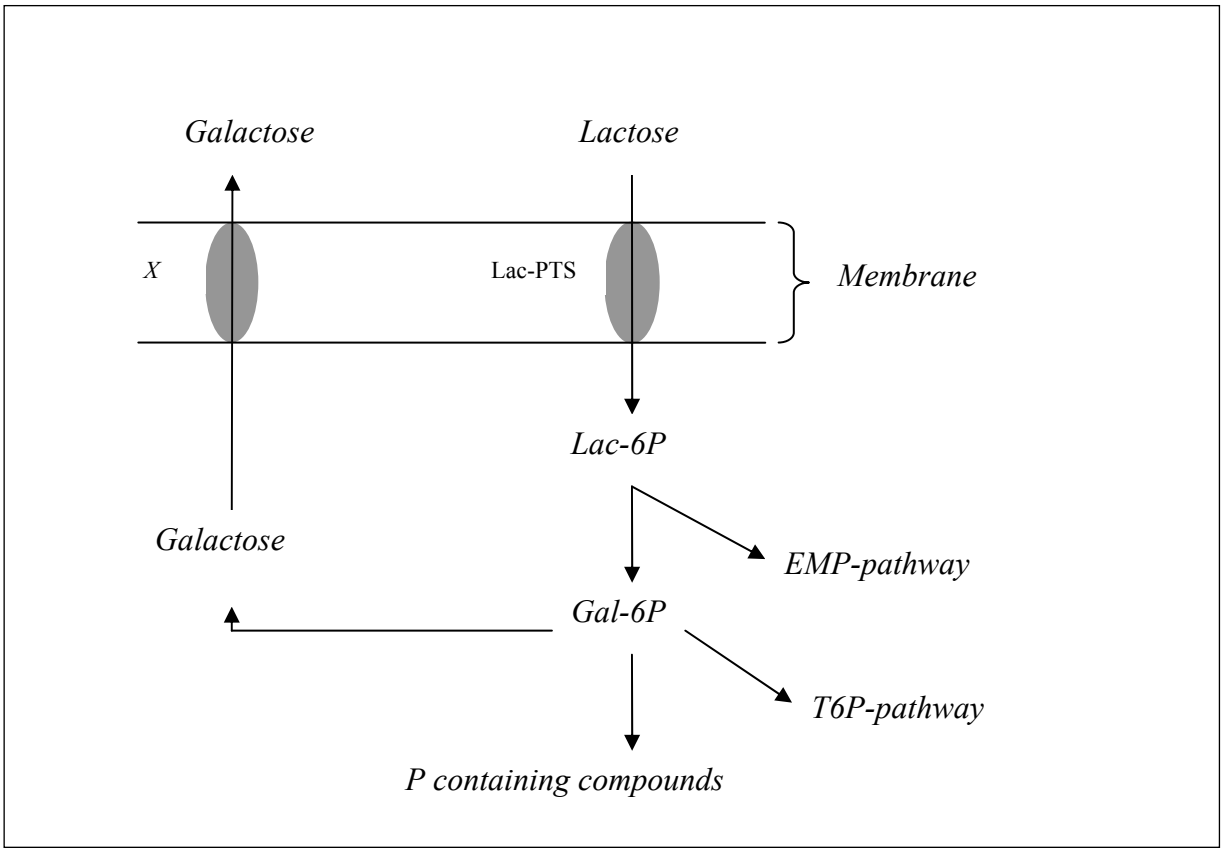

Figure 2.3 Schematic representation of dephosphorylation of Gal-6P and expulsion of galactose to the medium during lactose metabolism (Benthin *et al*., 1994).

Maltose fermentation among LAB has been studied most extensively in lactococci. Maltose is cleaved by a maltose phosphorylase into glucose and β-glucose-1-phosphate. The glucose moiety is used in glycolysis, whereas β-glucose-1-phosphate probably is a precursor for cell wall synthesis (Konings *et al*., 1989; Sjoberg and Hahn-Hagerdahl, 1989).

During sucrose fermentation, a permease system transports it into the cytoplasm where cleavage by sucrose hydrolase to glucose and fructose starts the metabolic process. Glucose and fructose enter the major pathways. In some lactococci, sucrose is transported by

sucrose PTS and sucrose-6-phosphate hydrolase cleaves the sucrose-6-phosphate to glucose-6-phosphate and fructose. The sucrose PTS and sucrose-6-phosphate hydrolase are induced by the presence of sucrose in the medium (Thompson and Chassy, 1981). Sucrose may also act as a donor of monosaccharides for exopolysaccharide formation in certain LAB. In dextran production by *Ln. mesenteroides*, sucrose is cleaved by a cell wall-associated enzyme, dextransucrase. The glucose moiety is used for dextran synthesis and fructose is fermented through the major pathways (Cerning, 1990; Salminen and Wright, 1998).

2.3.2 Nitrogen metabolism: proteolytic system

2.3.2.1 Proteolytic activity

LAB have a limited capacity to synthesize amino acids using inorganic nitrogen sources, therefore depend on preformed amino acids present in the growth medium as a nitrogen source. The conversion of peptides to free amino acids and the subsequent utilization of these amino acids is a central metabolic activity in LAB. However, the requirement for amino acids differs among the species and strain variations exist within species. Some strains of *Lactococcus* (*Lc*.) *lactis* ssp. *lactis* are in fact prototrophic for most amino acids, whereas *Lc. lactis* ssp. *cremoris* and *Lactobacillus* (*L.*) *helveticus* strains may require 13-15 amino acids (Chopin, 1993; Christensen *et al*., 1999). Growth on chemically defined minimal media is generally slow and it is clear that LAB have adapted to rich environments by developing a system to efficiently exploit the nitrogen sources present. One of the most extensively studied systems in this regard is the proteolytic system of dairy LAB, in particular that of *Lc. lactis*. This is due to the technological significance of this species in milk fermentation as a result of the proteolytic activity, which is necessary for appreciable and rapid growth in milk. The peptidase system is involved in the hydrolysis of exogenous peptides to obtain essential amino acids for growth and hydrolysis of peptides formed by housekeeping proteinases. The amino acids formed by this system can be utilized for processes such as protein synthesis, generation of metabolic energy, and recycling of reduced cofactors (Salminen and Wright, 1998; Christensen *et al*., 1999).

2.3.2.2 Proteolytic enzymes

The synthesis of proteolytic enzymes by starter cultures is a fundamental requirement for rapid acid production in milk or soymilk fermentation. These microorganisms possess a number of proteinases and peptidases which act in concert to hydrolyse milk or soymilk proteins to the free amino acids required for cell development.

The strategic location of these enzymes, in the cell wall and membrane structures and in the cytoplasm, governs enzyme access to the substrates (Thomas and Pritchard, 1987). The proteolytic system of starter bacteria is usually considered to consist of two functionally distinct classes of enzymes – proteinases, which catalyze the hydrolysis of native or denatured protein molecules, and peptidase, which catalyze the degradation of the smaller peptides produced by proteinases action (Law and Kolstad, 1983). The proteolytic system also includes the transport systems that translocate the breakdown products across the cytoplasmic membrane (Kunji *et al*., 1996).

The proteinase is clearly involved in the initial degradation of caseins, yielding a large number of different oligopeptides. The initial analyses of the casein breakdown products liberated by the proteinases have indicated that, with a few exceptions, only large peptides are formed (Pritchard and Coolbear, 1993). Consequently, further breakdown by extracellular peptidases was considered to be critical to fulfill the needs for essential and growth-stimulating amino acids (Kunji *et al*., 1996). Growth experiments in chemically defined media containing peptides of varying length have suggested that oligopeptide transport system (Opp) transports peptides up to lengths of 8 residues (Tynkkynen *et al*., 1993). Other experiments, in which translocation of peptides formed by the action of PrtP on β-casein was analyzed, indicate that oligopeptides consisting of up to 10 amino acids may be transported by oligopeptide transport system (Opp) (Kunji *et al*., 1996).

2.3.2.3 Proteinase of lactic acid bacteria and localization

The proteinases of many different LAB have been identified and characterized biochemically (Smid *et al*., 1991; Pritchard and Coolbear, 1993; Kok and De Vos, 1994). The degradation of caseins is initiated by a single cell wall-bound extracellular proteinase (PrtP). The external localization of proteinases is consistent with the finding that these are synthesized with a typical signal peptide sequence. This property has not been found in any of the peptidases analyzed so far (Kok and De Vos, 1994; Poolman *et al*., 1995). These results are supported further by biochemical and immunological data which indicate that the proteinases are present outside the cell, whereas most, if not all, peptidases are found in the cytoplasm (Kunji *et al*., 1996).

The extracellular location of PrtP is supported by the following findings: (i) the proteinase can be liberated from the cell-wall with minimal lysis by treatment of cells with Ca^{2+}-free buffers (Mills and Thomas, 1981), or lysozome (Coolbear *et al*., 1992), (ii) electron microscopy of immuno-gold labeled PrtP has confirmed a localization of the

proteinase in the cell (Hugenholtz *et al*., 1987), and (iii) the genes encoding proteinases specify a typical N-terminal signal sequence, which targets the protein to the outside of the cell (Kok and De Vos, 1994; Kunji *et al*., 1996).

2.3.2.4 Protein degradation

2.3.2.4.1 Specificity of proteinases

Degradation patterns of α_{s1}-, β- and κ-caseins have been grouped into two proteinase specificity- classes in lactococci, which are generally indicated as P_I and P_{III}. The primary substrates of P_I- type enzymes are β-casein, and to lesser extent, κ-caseins, while P_{III}-type enzymes degrade α_{s1}-, β- and κ-caseins (Pritchard and Coolbear, 1993). There are subtle changes on specificity as a result of minor genetic variations in the structural gene of PrtP. The *L. paracasei* enzyme differs from the *Lc. lactis* Wg2 proteinase in only two positions, that are regarded as important for substrate specificity and binding (Kunji *et al*., 1996). *L. delbrueckii* ssp. *bulgaricus* proteinase has distinct substitutions at position 138 (Gly), 166 (Val) and 747 (Gly), and similar residues at others. The serine-proteinase of *L. helveticus* L89 cleaves the same bonds as the lactococcal proteinases but differs in the relative amounts of fragments (Martin-Hernandez *et al*., 1994; Kunji *et al*., 1996).

Hydrolysis of β-casein by proteinases has been analyzed *in vitro* using purified enzymes of different *Lc. lactis* and *L. helveticus* strains (Monnet *et al*., 1989; Yamamoto *et al*., 1993). The proteolytic products were separated by high pressure liquid chromatography (HPLC) and the collected peptides were further purified and identified by Edman degradation and/or amino acid composition analysis. Where required, mass-spectrometric (MS) analysis of the purified peptides was performed. The results indicated that only part of β-casein was degraded and that relatively large fragments – only a few contain less than eight amino acid residue – were formed. However, inspection of the HPLC-profile showed that only the most abundant peptides were analyzed. Ten years ago, more than 95% of the peptides formed by the action of the proteinase of *Lc. lactis* ssp. *cremoris* Wg2 on β-casein were recovered using HPLC-ions-spray MS (Juillard *et al*., 1995a; Kunji *et al*., 1996). Apparently β-casein was degraded by PrtP into more than hundred different oligopeptides ranging from 4 to 30 residues, of which a major fraction fell in the range of 4 to 10 residues. Proteinase activity yielded undetectable amounts of di- and tripeptides, and only traces of phenylalanine were measured. More than 50% of the peptides originated from the C-terminal part of β-casein, while about half of the remaining peptides were derived from the 60-105 region (Kunji *et al*., 1996).

The major degradation products from κ-casein with the proteinase of *Lc. lactis* have been identified (Monnet *et al*., 1992). The inspection of the HPLC profiles of research papers suggests that the same peptides might be present in each hydrolysate, although in different amounts. The degradation of κ-casein yields a large number of small oligopeptides, which originate mainly from region 96-106 and the C-terminal part (Kunji *et al*., 1996).

Degradation of α_{s1}- and α_{s2}-caseins is hydrolyzed by P_{III}-type and intermediate-type proteinases of which 25 major oligopeptides were identified with about 50% originating from the C-terminal. Several different small oligopeptides are found in the hydrolysates of the various enzymes (Kunji *et al*., 1996).

2.3.2.4.2 Cleavage sites

Many research studies have attempted to define the specificity for proteinase activity based on statistical analysis of cleavage site residues and interaction of these residues with the PrtP areas (Monnet *et al*., 1989; Juillard *et al*., 1995a). It is apparent from inspection of results that the proteinases have very broad substrate specificity. Furthermore, the hydrolysis of casein by PrtP in a complex process, in which parameters such as refolding of the substrate after cleavage and aggregation of casein molecules may also attribute to a better accessibility of a particular loop for degradation (Kunji *et al*., 1996).

2.3.2.4.3 Peptidases of lactic acid bacteria and location

While the cellular location of the proteinase is undisputed, the localization of peptidases has been a subject to controversies (Kunji *et al*., 1996). Fractionation studies have suggested that some peptidases are present in cell-wall fractions. On the basis of the assumption that PrtP-generated casein degradation products are too large for transport; extracellular peptidases have been implicated in the proteolytic pathway (Law and Kolstad, 1983; Geis *et al*., 1985; Exterkate and De Veer, 1987; Smid *et al*., 1989; Blanc *et al*., 1993). However most biochemical, genetic and immunological data suggest an intracellular location for most, if not all, enzymes studied to date (Kunji *et al*., 1996; Christensen *et al*., 1999). In addition, more recent evidence for the ability of Opp to transport several large casein derived peptides negates the previous assertions that peptidase secretion would be required for liberation of essential amino acids (Kunji *et al*., 1996).

2.3.2.4.4 Peptidase specificity

The broad specificity aminopeptidase C (PepC) has been purified and characterized from strains of *L. helveticus, Lc. lactis* ssp. *cremoris*, *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus* (Wohlrab and Bockelmann, 1993). Significant activity of PepC on AA-βNAP substrates is reported for residues that are basic (Arg, His, and Lys), acidic (Glu and Asp), hydrophobic/uncharged (Ala and Leu), and aromatic (Phe). A similar level of activity is found for the corresponding AA-ρNA substrates, including Met- and Gly-ρNA. No PepC activity is detectable for Pro-ρNA, Pro-βNAP, Xaa-Pro-ρNA, or Xaa-Pro-βNAP substrates. Hydrolysis is also reported for a variety of di- and tripeptides with uncharged or basic residues in the amino terminal position (Kunji *et al*., 1996; Christensen *et al*., 1999).

The broad specificity of aminopeptidase N (PepN) has been purified and characterized from strains of *L. helveticus, Lc. lactis* ssp. *cremoris*, *L. delbrueckii* ssp. *bulgaricus*, *S. thermophilus* and *L. casei*. PepN is capable of cleaving N-terminal amino acids from a wide range of peptides differing in size and composition (Kunji *et al*., 1996; Christensen *et al*., 1999). In general, the activity of PepN on AA-ρNA substrates indicates the highest specificity for the basic amino acids Lys and Arg, followed by the hydrophobic /uncharged residues Leu and Ala. An appreciable activity is also observed for Met and Phe-ρNA, while in general low to undetectable activity is reported for Asp-, Glu-, and Gly-ρNA (Christensen *et al*., 1999). The enzyme of *Lc. lactis* shows a marked preference for dipeptides containing Arg as the N-terminal residue, but to a lesser extent of cleaving other residues such as Lys and Leu. Furthermore, an increase in activity is observed with increasing hydrophobicity of the C-terminal residue of the dipeptide Arg-X (Niven *et al*., 1995).

Aminopeptidase A (PepA) has been purified and characterized from *L. lactis* and *S. thermophilus* (Exterkate and De Veer, 1987**;** Bacon *et al*., 1994; Rul *et al*., 1995). PepA activity is essentially defined by the characteristic hydrolysis of N-terminal acidic amino acid residues. Rul *et al*. (1995) reported the hydrolysis of Glu- and Asp-ρNA, by PepA of *S. thermophilus* but with low activity for Glu- and Asp-βNAP. Hydrolysis of Glu-Xaa and Asp-Xaa dipeptides is observed when the C-terminus amino acid is basic (-Lys), uncharged (-Gly), hydrophobic/uncharged (-Ala, -Leu), polar/uncharged (-Ser), or aromatic (-Phe, -Tyr). The dipeptides Ser-Xaa are also hydrolyzed when the C-terminus amino acid is hydrophobic/uncharged (-Ala, -Leu) or aromatic (-Phe, -Tyr). PepA is also capable of releasing acidic residues from peptides as large as a decapeptides (Bacon *et al*., 1994).

Significant hydrolysis is not observed for di- and tripeptides with N-terminal amino acids from any other class (Christensen *et al*., 1999).

X-prolyl dipeptidyl aminopeptidase, PepX, has been purified and characterized from strains of *L. helveticus, Lc. lactis* ssp. *cremoris*, *L. delbrueckii* ssp. *bulgaricus*, *S. thermophilus*, *L. acidophilus* and *L. casei*. PepX is capable of cleaving Xaa-Pro dipeptides from the N-terminus of peptides (Houbart *et al*., 1995; Gatti *et al*., 2004; Pan *et al*., 2005). The highest activities reported for PepX on Xaa-Pro-ρNA substrates are when the N-terminal residues are uncharged (Ala-, Gly-) or basic (Arg-). While liberation of amino acids from dipeptides does not occur, PepX releases Xaa-Pro dipeptides from three to seven amino acid residues (Christensen *et al*., 1999). PepX is also capable of hydrolyzing Pro-Pro-$(Xaa)_n$ substrates, but little or no hydrolysis is observed for Xaa-Pro-Pro (including when Xaa is Pro) (Booth *et al*., 1990; Miyakawa *et al*., 1994; Pan *et al*., 2005).

Dipeptidase, PepV, has been purified and characterized from strains of *L. helveticus, Lc. lactis* ssp. *cremoris*, *L. delbrueckii* ssp. *bulgaricus*, and *L. casei*. (Wohlrab and Bockelmann, 1992; Gobbetti *et al*., 1996; Fernandez-Espla and Martin-Hernandez, 1997). PepV is considered a broad specificity dipeptidases. No hydrolysis of AA-ρNA substrates and no significant hydrolysis of Pro containing peptides is reported (Christensen *et al*., 1999). A wide range of dipeptides are hydrolyzed containing basic (Arg-, His-, Lys-), hydrophobic/uncharged (Ala-, Ile-, Leu-, Val-), aromatic (Phe-, Tyr-), and Met residues in the N-terminal position. In contrast, PepV generally does not hydrolyze dipeptides containing Gly as the N-terminal residue (Christensen *et al*., 1999).

The aminopeptidase, PepP, has been purified and characterized from *Lc. lactis* ssp. *cremoris* and liberates the N-terminal amino acid from peptides with general specificity for Xaa-Pro-Pro- $(Yaa)_n$ sequences (Mars and Monnet, 1995). Relatively high activity was observed with peptides ranging from three to nine residues, but the dipeptides tested were not hydrolysed. The rate of hydrolysis was highest for the pentapeptides Arg-Pro-Pro-Gly-Phe and Leu-Pro-Pro-Ser-Arg (Mars and Monnet, 1995).

The tripeptidase, PepT, has been purified and characterized from *Lc. lactis* ssp. *cremoris* (Mars and Monnet, 1995). PepT hydrolyses tripeptides with a wide range of amino acids including substrates comprised of hydrophobic/uncharged, aromatic, basic, acidic and sulphur-containing residues. Hydrolysis is observed for Pro-Gly-Gly and Leu-Xaa-Pro (-Gly-, -Ala-), but not for any Xaa-Pro-Yaa substrates tested. In accordance with the strict substrate size specificity of PepT, no activity has been reported for any di-, tetra-, or larger oligopeptides (Christensen *et al*., 1999).

Endopeptidase, PepE, has been purified and characterized from strains of *L. helveticus*. PepE was initially identified on the basis of hydrolysis of the N-terminal and C-terminal blocked substrate, N-benzoyl-Phe-Val-Arg-ρNA (Fenster *et al*., 1997). Purified PepE was also capable of hydrolyzing Met-enkephalin (Tyr-Gly-Gly-Phe-Met) at the Gly_3-Phe_4 bond and Bradykinin (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg) at the Gly_4-Phe_5 bond. No activity was reported with β-casomorphin, N-benzoyl-Val-Gly-Arg-ρNA, or with Arg-, Phe-, Pro-, Lys-, Gly- or Val- ρNA (Christensen *et al*., 1999).

Endopeptidase, PepO, has been purified and characterized from *Lc. lactis* ssp. *cremoris* (Tan *et al*., 1991; Stepaniak and Fox, 1995; Lian *et al*., 1996). PepO is reported to hydrolyze oligopeptides ranging from five (Met- and Leu-enkephalin) to thirty-five residues (αs1-casein f165-199) (Tan *et al*., 1991; Stepaniak and Fox, 1995; Lian *et al*., 1996). Hydrolysis also occurs with bradykinin, angiotensin, neurotensin, and insulin β-chain. Although PepO has activity with several large casein derived fragments, the native caseins are not detectably hydrolyzed (Tan *et al*., 1991; Stepaniak and Fox, 1995).

2.4 Viability of probiotic bacteria

In order to obtain the desired health effects, probiotic bacteria must be able to grow in milk/soymilk and survive in sufficient numbers. It has been suggested that probiotic organisms should be present in a food at a minimum concentration of 10^5-10^6 cfu/g (Kurmann and Rasic, 1991; Rybka and Fleet, 1997; Gomes and Malcata, 1999), or the daily intake should be about 10^8 cfu/g. Such high numbers have been recommended to compensate for possible losses in the numbers of the probiotic organisms during passage through the stomach and intestine. Despite the importance of viability of probiotic microorganisms (*L. acidophilus* and *Bifidobacterium* sp.), surveys have shown poor viability in yoghurts (Shah *et al*., 1995; Dave and Shah, 1997; Rybka and Fleet, 1997; Shah, 2000b). Therefore, in order to provide the therapeutic benefits, attempts to increase viability in dairy and soy products have drawn the attention of researchers in recent years. On the other hand, there is much evidence to show that *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus* survive at high concentration (> 10^7 cfu/g) in yoghurts after manufacture until the time of consumption (Rohm *et al*., 1990) in comparison to probiotic microorganisms. Several factors have been claimed to affect the viability of probiotic cultures in fermented milk/soy products.

2.4.1 Factors affecting viability of probiotic bacteria

Several factors, including the strains selected, interactions between species present, acidity, pH and hydrogen peroxide due to bacterial metabolism, have been identified to affect the viability of probiotic microorganisms during manufacture and storage of yoghurt or soy yoghurt (Lankaputhra *et al*., 1996; Dave and Shah, 1997). Other factors such as storage temperature, oxygen content, concentrations of acetic and lactic acids, nutrients limitations in milk/soymilk to sustain growth, growth promoters and inhibitors, inoculation level, fermentation time and post-acidification have also been suggested to affect viability of probiotic organisms in yoghurt or soy yoghurt (Samona and Robinson 1994; Rybka and Kailsapathy, 1995; Dave and Shah, 1997; Dave and Shah, 1998; Shah, 2000b; Shah and Ravula, 2000; McComas and Gilliland, 2003; Tamime, 2005).

The improvement of survival and viability may be achieved by either appropriate culture selection (Lourens-Hattingh and Viljoen, 2001; Tuomola *et al*., 2001), microencapsulation (Capela *et al*., 2006), supplementation of milk and soymilk with nutrients (Dave and Shah, 1998; McComas and Gilliland, 2003) or use of growth enhancers such as prebiotics (Bruno *et al*., 2002; Corcoran *et al*., 2004; Capela *et al*., 2006).

2.4.2 Strategies for improving viability of probiotic organisms

2.4.2.1 Supplementation of milk and soymilk with nutrients

Growth of some probiotic bacteria (*L. acidophilus* and *Bifidobacterium* ssp.) was substantially improved in milk supplemented with dairy and non-dairy ingredients whey powder (WP); whey protein concentrates (WPC); acid casein hydrolysate (ACH); and whey protein hydrolysate (WPH) (Dave and Shah, 1998; McComas and Gilliland, 2003). This was not surprising because poor proteolytic activity of probiotic bacteria limits the growth in milk/soymilk (Klaver *et al*., 1993). The added ingredients apparently provided a readily available source of peptides and amino acids as growth factors required for growth of probiotic bacteria, consequently shortening the incubation time considerably (Dave and Shah, 1998).

2.4.2.2 Microencapsulation

Microencapsulation of probiotics can be carried out with natural polymers to reduce cell losses during processing and storage. It can also be used to regulate fermentation by lactic acid producing starter culture. Microencapsulation is a process where the cells are retained within an encapsulating membrane in order to reduce the cell injury or cell loss.

The use of gelatine or vegetable gum as encapsulating materials has been reported to provide protection to acid sensitive probiotic organisms (Capela *et al*., 2006). Microencapsulation with sodium alginate has been shown to improve viability of probiotic organisms in freeze-dried yoghurt stored at 21^{o}C (Capela *et al*., 2006). Alginate is non-toxic and may be safely used in foods. Encapsulated probiotic organisms when incorporated in fermented frozen dairy desserts or yoghurt showed improved viability in comparison to non-encapsulated control organisms (Ravula and Shah, 2000; Capela *et al*., 2006).

2.4.2.3 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 (Ziemer and Gibson, 1998). This selectivity has been demonstrated for *Bifidobacterium*, whose growth may be promoted by the uptake of substances such as fructo-oligosaccharrides, transgalactosylated oligosaccharides and soybean oligosaccharides (Schrezenmeir and Vrese, 2001). Beside their prebiotic properties, certain oligosaccharides have shown a number of functional effects on the GIT physiology including reduced-fat-and cholesterol absorption, modulation of microbial proliferation, thus subsequently reducing intestinal disturbances, cardiovascular disease and intestinal cancer (Ziemer and Gibson, 1998).

A range of oligosaccharides has been assessed for prebiotic properties (Kaplan and Hutkins, 2000; Roberfroid, 2001) with inulin and other fructo-oligosaccharides frequently employed in studies as they resist digestion by gastric acid and pancreatic enzymes *in vivo* (Cummings *et al*., 2001). In most instances, *Bifidobacterium* sp. have been the main focus of research (Shin *et al*., 2000; Bruno *et al*., 2002; Akalin *et al*., 2004), although *Lactobacillus* has been used widely in dairy products and should deserve attention due to their established health-promoting effects (Shah, 2000a; 2001).

Inulin is a non-digestible fructan frequently used as a functional food ingredient that offers a unique combination of interesting nutritional properties and important technological benefits (Suzuki and Chatterton, 1993). Another added benefit of inulin that is often capitalized on in yoghurt is the prebiotic effect, which may serve to reinforce or enhance the action of probiotic cultures typically added to yoghurt (Niness, 1999). Inulin is present in many regularly consumed vegetables, fruits and cereals, including leek, onion, garlic, wheat, chicory, artichoke, and banana.

The molecular structure of inulin is shown in Figure 2.4a. A glucose molecule typically resides at the end of each fructose chain and is linked by an α(1-2) bond, as in sucrose. The chain lengths of these fructans range from 2 to 60 units, with an average DP of ~10 (De Leenheer and Hoebregs, 1994; IUB-IUPAC Joint Commission on Biochemical Nomenclature, 1982; VanHaastrecht, 1995). The unique aspect of inulin is its β(2→1) bonds connecting fructose monomers and thus preventing digestion by enzyme, and are responsible for its reduced caloric value and dietary fiber effects (Niness, 1999).

In contrast to inulin, amylose maize starch (Hi-maize®) is composed solely of glucose monomers and exhibits a range of qualities that strongly indicate its capability to act as a prebiotic in foods (Haynes and Playne, 2002; Brown *et al*., 1998). Starch, which is the major dietary source of carbohydrates, is the most abundant storage polysaccharide in plants, and occurs as granules in the chloroplast of green leaves and the amyloplast of seeds, pulses, and tubers (Ellis *et al*., 1998).

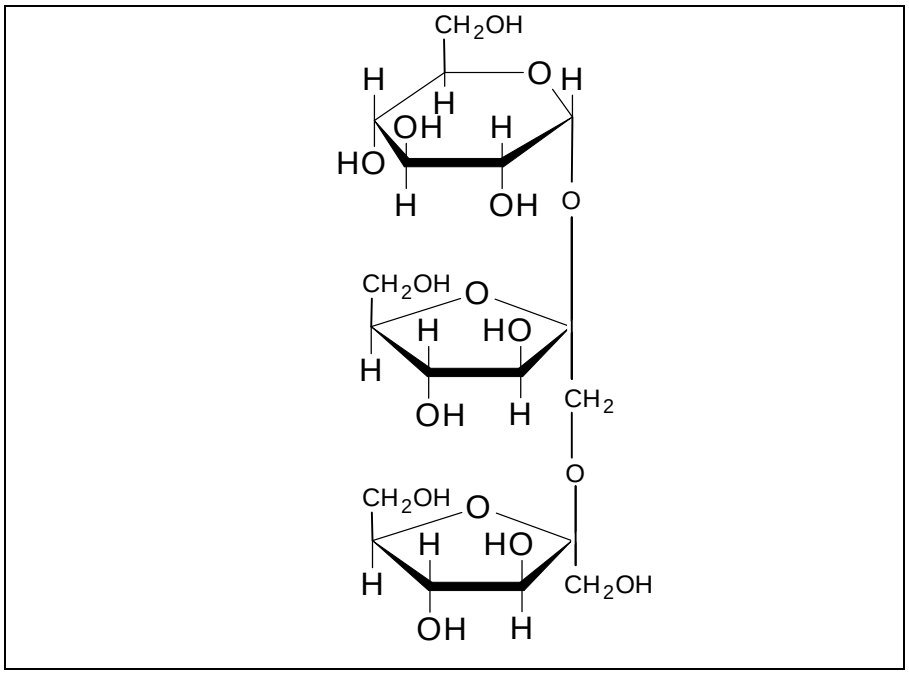

Figure 2.4 a Chemical structure of inulin (De Leenheer and Hoebregs, 1994).

Chemically, starch is a polysaccharide, composed of a number of monosaccharides or glucose molecules linked together with α-D-(1→4) linkages (Figure 2.4 b). The starch amylose, is a linear polymer in which glucose residues are α-D-(1→4) linked and this linear molecular structure can form tightly packed granules which is insoluble and hard to digest (British Nutrition Foundation, 1990; Sajilata *et al*., 2006). Starch, commonly referred to as

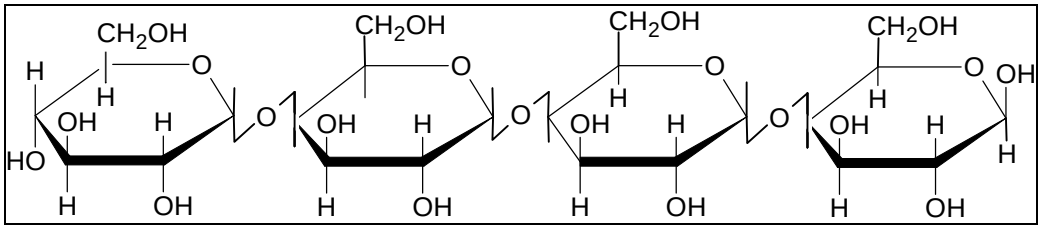

Figure 2.4b Section of amylose molecule (British Nutrition Foundation, 1990)

resistant starch, has been suggested for use as a prebiotic in probiotic preparations to promote the growth of some beneficial microorganisms such as *Bifidobacterium* (Brown *et al*., 1996; Cummings and Englyst, 1991).

Apart from serving as a growth promoter, when allowed to ferment Hi-maize, various species of bacteria produce a range of potentially beneficial short-chain fatty acids (SCFA) including acetate, propionate, and butyrate (Brown, *et al*., 1998; Le Leu *et al*., 2005). Among the SCFA that are produced in the colon, butyrate is considered to play a role in regulating intestinal cell functions and growth by suppressing tumor cells and decreasing the proliferation of colonic mucosal cells (Johnson and Gee, 1996). Food applications of resistant starch are of interest to product developers and nutritionists for two reasons: (i) fibre-fortification and the potential physiological benefits of resistant starch, which may be similar to fibre, and (ii) unique functional properties, yielding high quality products not attainable otherwise with traditional insoluble fibres. Its physical properties, particularly low water holding capacity, allow it to be a functional ingredient that provides good handling during processing and improved texture in the final product (Yue and Waring, 1998).

2.5 The concept of synbiotic

The combination of suitable probiotics and prebiotics enhances survival and activity of the organism, for example a FOS in conjunction with a *Bifidobacterium* strain or lactitol in conjunction with *Lactobacillus* (Gibson and Roberfroid, 1995). The combination of prebiotic and probiotic has synergistic effects because in addition to promoting growth of existing strains of beneficial bacteria in the colon, synbiotics also act to improve the survival, implantation and growth of newly added probiotic organisms. The synbiotic concept has been widely used by dairy drink and yoghurt manufacturers in Europe such as Aktifit (Emmi, Switzerland), Proghurt (Ja Naturlich Naturprodukte, Austria), Vifit (Belgium and UK) and Fysiq (The Netherlands) (Niness, 1999).

2.6 Probiotic effect

Since Metchnikoff's era, a number of health benefits has been contributed to products containing probiotic organisms. While some of these benefits have been well documented and established, others have shown promising potential in animal models. However, human studies are required to substantiate these claims. More importantly, health benefits provided by probiotic bacteria are strain specific; therefore, there is no universal strain that would provide all proposed benefits, not even strains of the same species. Moreover, not all the strains of the same species are effective against a defined health conditions. The strains, *L. rhamnosus* GG (Valio), *S. cerevisiae* Boulardii (Biocodex), *L. casei* Shirota (Yakult), *B. animalis* Bb-12 (Chr. Hansen), *L. acidophilus* LAFTI® L10, *B. animalis* ssp. *lactis* LAFTI® B94 and *L. casei* LAFTI® L26 (DSM Food Specialties) (Crittenden *et al*., 2005) are certainly the most investigated probiotic organisms with the established human health efficacy data against management of lactose malabsorption, rotaviral diarrhoea, antibiotic-associated diarrhoea, and *Clostridium difficile* diarrhoea.

2.7 Functionality of bioactive peptides

Peptides with various biological activities can be generated during milk fermentation with proteolytic starter cultures. As a result, peptides with wide functionalities can be found in the end-products of fermentation of dairy foods (Gobbetti *et al*., 2004; Korhonen and Pihlanto, 2003). There is now a considerable amount of scientific data to demonstrate that a wide range of milk peptides can regulate specific physiological functions in experimental animals and humans. These functions relate to general health conditions or a reduced risk of certain chronic diseases of the nervous, cardiovascular, digestive and immune systems.

Angiotensin-converting enzyme (ACE) is a major target for cardiovascular therapies and ACE inhibitors have been on the market for more than 20 years (Acharya *et al*., 2003). ACE is a central component of the rennin-angiotensin system (RAS), which controls not only blood pressure, and fluid and electrolyte homeostasis, but also renal and vascular function and myocardial remodeling (Xie, 1990; Turner and Hooper, 2002). As such, inhibitors of the RAS have found a widespread application in cardiovascular diseases, and current ACE inhibitors have a broad range of licensed indications, ranging from mild hypertension to post-myocardial infarction (Zaman *et al*., 2002). Current-generation ACE inhibitors are widely used for cardiovascular diseases, including high blood pressure, heart failure, heart attack and kidney failure, and have combined annual sales in excess of US $6 billion. However, the use of these ACE inhibitors is hampered by common side effects

(Messerli, 1999). Moreover, it has been known that ACE actually consists of two parts comprising an N- and C-domains that have different functions (Acharya *et al*., 2003). Each domain contains an active site with similar but distinct substrate specificities and chloride-activation requirements (Turner and Hooper, 2002). Therefore, if domain-selective inhibitors can be designed, this could produce next-generation drugs with altered safety and efficacy profiles.

2.7.1 Production of bioactive peptides

Peptides with ACE-inhibitory (ACE-I) activity have already been isolated from different food proteins (Ariyoshi, 1993; Yamamoto, 1997). These biologically active peptides could represent a healthy and natural alternative for the ACE-I drugs. Milk proteins are a rich source of bioactive peptides. These peptides have hormone- or drug-like activity that eventually modulate physiological function through binding interactions to specific receptors on target cells leading to induction of physiological responses (Fitzgerald and Murray, 2006). Bioactive peptides can be produced through enzymatic hydrolysis by digestive enzymes of whole protein molecules. Many of the known bioactive peptides have been produced using enzymes from GIT, usually pepsin and trypsin (Korhonen and Pihlanto, 2006). ACE-I peptides and calcium-binding phosphopeptides (CPPs) are most commonly produced by trypsin (Meisel and Fitzgerald, 2003; Fitzgerald *et al*., 2004; Gobbetti *et al*., 2004; Vermeirssen *et al*., 2004). Other digestive enzymes and different enzyme combinations of proteinase including alcalase, chymotrypsin, pancreatin, pepsin and thermolysin as well as enzymes from bacteria and fungal sources have been used to produce bioactive peptides from various proteins as reviewed in recent articles (Korhonen and Pihlanto, 2006).

Bioactive peptides can also be generated by starter and non-starter bacteria used in the manufacture of fermented dairy/soy products during proteolysis. The proteolytic system of LAB, *L. helveticus, Lc. lactis* ssp. *cremoris* and *L. delbrueckii* ssp. *bulgaricus* is well characterized (Wohlrab and Bockelmann, 1992; Law and Haandrikman, 1997). This system consists of proteinase and peptidases (Kunji *et al*., 1996; Christensen *et al*., 1999). The release of bioactive peptides during fermentation of milk using different proteolytic microorganisms or proteolytic enzymes derived from microorganisms is presented in Table 2.1. These bioactive peptides released from milk proteins through proteolytic activities of microorganisms include ACE-I or antihypertensive peptides, immunomodulatory, antioxidative and antimicrobial peptides (Smacchi and Gobbetti, 2000; Shah, 2000c;

Gobbetti *et al*., 2004; Silva and Malacata, 2005; Korhonen and Pihlanto, 2006). *Lactobacillus helveticus*, capable of releasing ACE-I peptides, is widely used in the manufacture of traditional fermented milk products, such as Calpis®. The best known ACE-I peptides, including VPP and IPP, have been identified in milk fermented with *L. helveticus* strains (Nakamura *et al*., 1995; Seppo *et al*., 2003). Gobbetti *et al*. (2000) showed the production of ACE-I peptides in milk fermented separately with *Lb. delbrueckii* ssp. *bulgaricus* strains and *Lactococcus lactis* ssp. *cremoris*, for 72 h. The most inhibitory fractions of the fermented milk mainly contained β-casein-derived peptides with inhibitory concentration (IC_{50}) values ranging from 8.0 to 11.2 μg/mL (Korhonen and Pihlanto, 2006). Schieber and Bruckner (2000) identified and characterized oligo- and polypeptides isolated from yoghurt with several possible precursors of peptides with known biological activity.

The sequences Ala-Val-Pro-Tyr-Pro-Gln-Arg and Tyr-Gln-Glu-Pro-Val-Leu from β-casein have been demonstrated to inhibit the ACE, and therefore, have an antihypertensive effect. The use of mixed starter cultures with enhanced proteolytic activities might lead to the formation of peptides with known or novel bioactivities from milk proteins (Schieber and Bruckner, 2000; Ashar and Chand 2003). More recently, Ashar and Chand (2004) identified ACE-I peptide from milk fermented with *Lb. delbrueckii* ssp. *bulgaricus*. The peptide showed the sequence Ser-Lys-Val-Tyr-Pro-Phe-Pro-Gly-Pro-Ile from β-casein with IC_{50} value of 1.7 mg/mL. In combination with *S. thermophilus* and *Lactococcus lactis* ssp. *lactis* biovar diacetylactis, a peptide structure with a sequence of Ser-Lys-Val-Tyr-Pro was obtained from β-casein with IC_{50} value of 1.4 mg/mL. Both peptides were markedly stable to digestive enzymes, acidic and alkaline pH, as well as during storage at 5 and 10^{o}C for 4 days (Korhonen and Pihlanto, 2006). The size of active sequences may vary from 2 to 20 amino acid residues, and many peptides are known to have multifunctional properties (Meisel and Fitzgerald, 2003). However, most of the reported ACE-I peptides are usually short peptides with a proline residue at the carboxyl terminal end. Also, proline is known to be resistant to degradation by digestive enzymes and may pass from the small intestines into the blood circulation in the sequence of short peptides (Yamamoto *et al*., 2003). This hypothesis is supported by a recent study by Pan *et al*. (2005) who hydrolysed skimmed milk with a cell-free extract of *L. helveticus* JCM1004 and purified the antihypertensive tripeptides, VPP and IPP. The IC_{50} values of the peptides were 9.13 ± 0.21 and 5.15 ± 0.17 μM, respectively (Korhonen and Pihlanto, 2006).

Table 2.1 Bioactive peptides released from milk proteins by various microorganisms and microbial enzymes

Microorganisms	Precursor protein	Peptide sequence	Bioactivity	References
L. helveticus *S. cerevisiae*	β- CN [*], κ-CN	Ile-Pro-Pro, Val-Pro-Pro	ACE [#] inhibition, antihypertensive	Nakamura *et al*. (1995) Takano (1998)
L. helveticus JCM1004 cell-free extract	Skim milk hydrolysate	Ile-Pro-Pro, Val-Pro-Pro	ACE inhibition, antihypertensive	Pan *et al*. (2005)
Lactobacillus GG Enzymes + pepsin and trypsin	β- CN, α_{s1}- CN	Tyr-Pro-Phe-Pro, Ala-Val-Pro-Tyr-Pro-Gln-Arg, Thr-Met-Pro-Leu-Trp	ACE inhibition	Rokka *et al*. (1997)
L. helveticus CP790 proteinase	β- CN	Lys-Val-Leu-Pro-Val-Pro-Gln	ACE inhibition	Maeno *et al*. (1996)
L. helveticus CPN4	Whey proteins	Tyr-Pro	ACE inhibition	Yamamoto *et al*. (1999)
L. delbrueckii ssp. *bulgaricus* SS1, *Lactococcus lactis* ssp. *cremoris* FT4	β-cn, κ- CN	Many fragments	ACE inhibition	Gobbetti *et al*. (2000)
L. delbrueckii ssp. *bulgaricus*	β- CN	Ser-Lys-Val-Tyr-Pro-Phe-Pro-Gly-Pro-Ile	ACE inhibition	Ashar and Chand (2004)
S. thermophilus + *Lc. lactis* ssp. lactis biovar. *diacetylactis*	β- CN	Ser-Lys-Val-Tyr-Pro	ACE inhibition	Ashar and Chand (2004)
Lactococcus lactis	α_{s1}-CN, α_{s2}- CN, κ- CN	Many fragments	ACE inhibition	Minervini *et al*. (2003)
Lb. helveticus NCC 2765	β- CN	Tyr-Pro-Phe-Pro-Glu-Pro-Ile-Pro-Asn	Opioid	Meisel and Frister (1989)
Commercial products + digestion	α_{s1}- CN	Thr-Thr-Met-Pro-Leu-Trp	ACE inhibition, immunomodulation	Maruyama *et al.* (1987)

CN [*] = casein; ACE [#] = angiotensin I- converting enzyme.

Maruyama and Suzuki (1982) reported that tryptic hydrolysates of casein inhibited the *in vitro* activity of ACE. Those peptides derived from casein, known as casokinins, corresponded to f23-24, f23-27 and f194-199 of bovine αs1-casein B, as well as to f177-183 and f193-202 of bovine β-casein (Maruyama and Suzuki, 1982; Maruyama *et al*., 1987). Fuglsang *et al*. (2003) studied a total of 26 strains of wild-type LAB, mainly belonging to *Lc. lactis* and *L. helveticus*, for their ability to produce a milk fermentate with ACE-I activity. They found that most LAB produced ACE inhibitors in varying amounts during milk fermentation which varied with strains.

Nakamura *et al*. (1995) purified and characterized ACE-I peptides from milk fermented with *L. helveticus* and *S. cerevisiae* at 37^{o}C for 24 h. Two ACE-I peptides, VPP and IPP, were identified and after purification, 74.4% of the initial ACE- I activity was recovered with these peptides suggesting that most of the ACE-I activity of the sour milk is attributed to these two peptides produced during fermentation. Amino acid sequences of VPP and IPP are found in the primary structure of bovine β-casein (84-86), β-casein (74-76), and κ-casien (108-110), respectively. The IC_{50} values of VPP and IPP were 9 and 5 μM, respectively (Nakamura *et al*., 1995). Several milk casein-derived peptides are able to lower blood pressure and various peptides that consist of 6-12 amino acid residues have been reported to possess antihypertensive effects in spontaneously hypertensive rats (SHR) following single oral administration (Karaki *et al*., 1990; Maeno *et al*., 1996).

2.7.2 Effect of inhibition of ACE on cardiovascular system

Blood pressure regulation is partially dependent on the rennin-angiotensin system; rennin acts on the angiotensinogen and release angiotensin I, which is further converted into the active peptide hormone angiotesin II, a vasoconstrictor, by the ACE. At the same time, ACE inactivates bradykinin, a vasodilator. It further increases the production of aldosterone, which decreases the renal output while increasing water retention (Maruyama *et al*., 1987). Inhibition of this enzyme can exert an antihypertensive effect. To exert an antihypertensive effect after oral ingestion, active peptides must be absorbed in an intact form from the intestine into the blood and further resist the degradation by plasma peptidases in order to reach the target sites.

In a placebo-controlled trial with mildly hypertensive subjects, a significant reduction in blood pressure was recorded after daily ingestion for 4 weeks of 95 mL of “Calpis” sour milk containing the potent ACE-inhibitory peptides, VPP and IPP. The ingested dose of these

peptides was 2.6 mg per day. Blood pressure was reverted gradually to pre-trial level after the intervention period ceased. No major changes in blood pressure were observed in the placebo group (Hata *et al*., 1996; Yamamoto *et al*., 2003). Similar results were reported by Seppo *et al*. (2002) and Seppo *et al*. (2003) with the "Evolus" product in two double-blinded, placebo-controlled studies with mild hypertensive subjects who ingested 150 mL of the product daily. "Evolus" was found to decrease both systolic and diastolic blood pressure during the 8-week and 21 week treatment periods, respectively. No such effect was observed in subjects with normal blood pressure.

2.8 Isoflavone

Isoflavones are part of the diphenol compounds, called phytoestrogens, which are structurally and functionally similar to estradiol, the human estrogen (Figure 2.5), but much less potent. Because of this similarity, isoflavones are suggested to have preventive effects for many kinds of hormone-dependent diseases (Uzzan and Labuza, 2004). Soybean and soy-food derived isoflavones are found as four chemical forms, including aglycones, malonyl- acetyl- and β-glucoside conjugates. The biologically active, estrogen-like isoflavone compounds are the aglycone configurations of genistein, daidzein and glycitein (Figure 2.6a) (Tsangalis *et al*., 2002). The isoflavone consumption has been linked to reduced risk of most hormone-associated health disorders prevalent in current Western civilizations (Setchell and Lydeking-Olsen, 2003). Asian populations, with their high intake (50 – 70 mg/d) of soy-derived isoflavones, have been recognized to have the lowest incidence of osteoporosis, postmenopausal symptoms, and mortality from cardiovascular disease and cancer (Nagata *et al*., 1998).

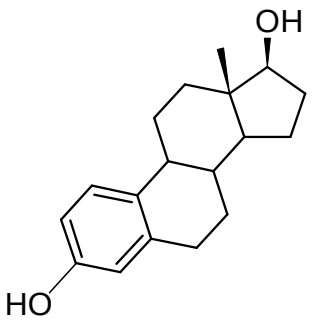

Figure 2.5 Estradiol, the human estrogen (Setchell *et al*., 2002)

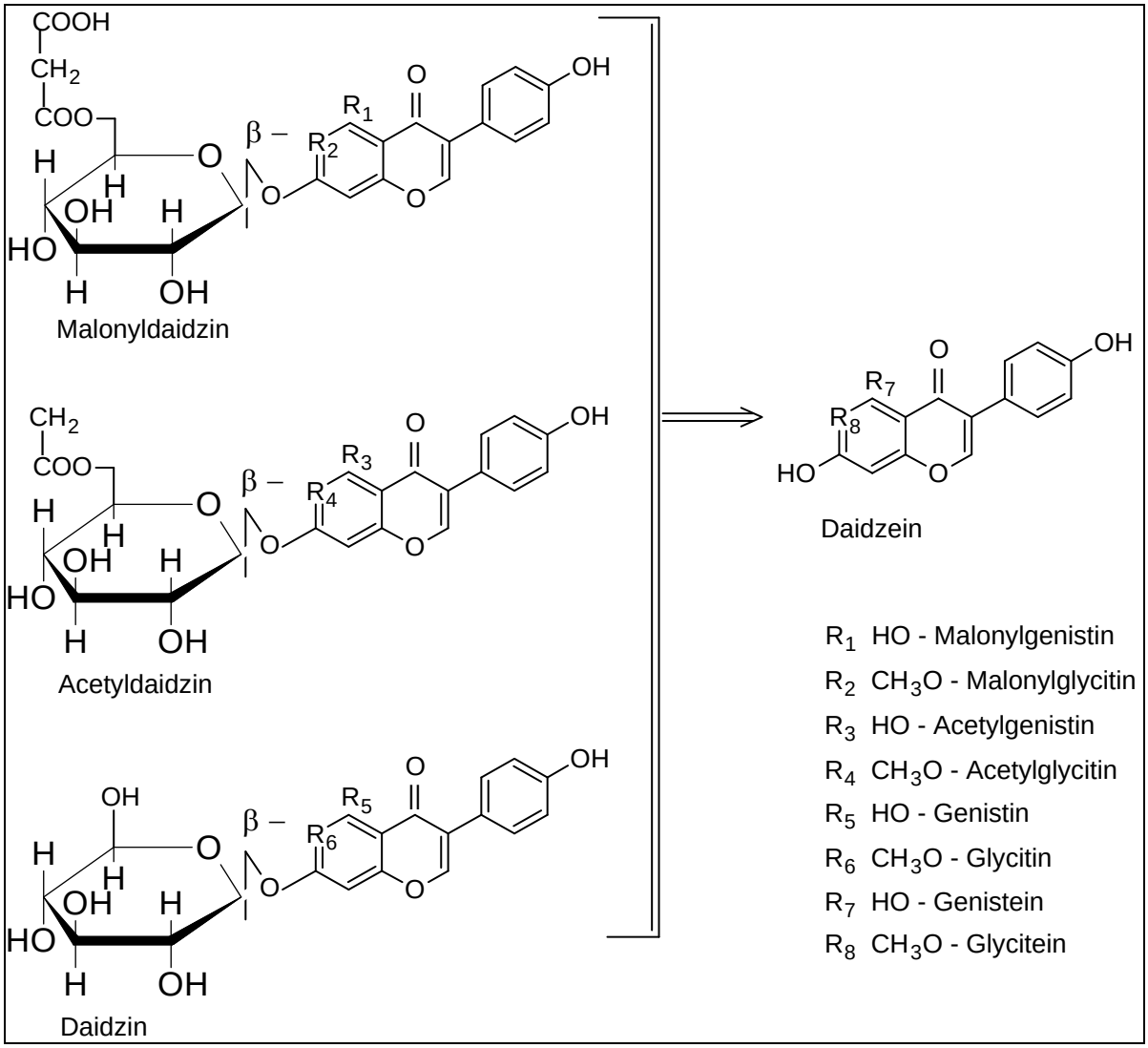

Figure 2.6 a Structural representation of isoflavone compounds and transformations as a result of bacterial-induced enzymatic hydrolysis of β-glycosidic linkage (Tsangalis *et al*., 2002).

2.8.1 Biotransformation of soy isoflavones

Isoflavones are commonly found in soybeans and nonfermented soy foods as biologically inactive glycoside (or glucoside) conjugates, which comprise 80 to 95% of the isoflavone content (King and Bignell, 2000). Isoflavones have been studied extensively and are found to be metabolically stable. Recent research has shown that isoflavone aglycones in soy foods are absorbed faster and in higher amounts than their respective glucosides in humans (Setchell *et al*., 2001). Twelve forms of isoflavones are known in soybeans and soy products, including 3 "free" forms, called aglycones (genistein, daidzein, and glycitein) and 3 conjugated forms to each aglycone called glucosides. The conjugated forms have an additional glucose moiety, which could be free of other groups (β-glucosides; namely, genistin, daidzin, and glycitin) or could be bound to either an acetyl group (6"- O-acetylglucosides) or a malonyl group (6"- O-malonylglucosides) (Uzzan and Labuza, 2004).

It has been suggested that intestinal microflora play an important role in the metabolism and bioavailability of isoflavones by hydrolyzing the glucoside components via β-glucosidase in the jejunum, releasing the bioavailable and bioactive aglycone form (Setchell, 2000). Furthermore, intestinal bacteria chemically change daidzein to equol (Setchell and Cassidy, 1999). The aglycones are released and further metabolism of daidzein and genistein takes place (Figure 2.6 b). Biotransformations by intestinal bacteria include dehydroxylation, reduction, C-ring cleavage and demethylation. These reactions take place distally and presumably in the colon. Glycitin, the 6-methoxy analogue of daidzin, is found in high proportions in soy germ but is a minor component of most soy foods. Its glycoside is readily hydrolysed to release glycitein but the close proximity of the 6-methoxyl to the 7-hydroxyl sterically hinders its demethylation. Thus, glycitein is not converted to daidzein to any appreciable extent and is therefore not a precursor of equol. The formation of equol from daidzein occurs via a pathway that involves the formation of the intermediate dihydrodaidzein (Figure 2.6 b) (Setchell *et al*., 2002).

More recent studies have indicated that the extent of metabolic transformation of dietary isoflavones is more complex than originally thought. A detailed qualitative analysis of human urine revealed many other diphenolic metabolites that represent intermediates in the biotransformation of diadzein and genistein, many of which can only be successfully quantified by mass spectrometry (Setchell, 1998). Some strains of *Bifidobacterium* are known to produce

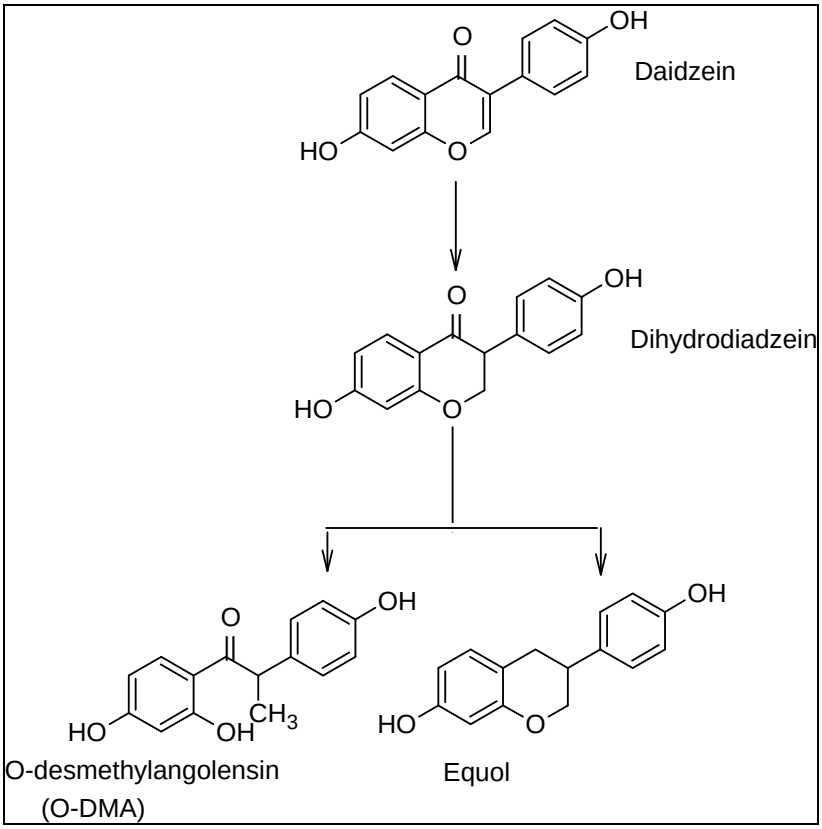

Figure 2.6 b Metabolism of diadzein (King *et al*., 1998)

β-glucosidase required to hydrolyse isoflavone glucosides to bioactive aglycones in soymilk (Tsangalis et al., 2002).

2.8.2 Biological properties of isoflavones

The incidence of sudden decline in estrogen levels after menopause coincides with acceleration of several aging processes (Yaffe *et al*., 1998). On average, bone mineral density (BMD) decreases and cognitive functions decline, whereas total cholesterol and low-density lipoprotein cholesterol (LDL-C) level increases. It has been suggested that postmenopausal estrogen therapy might counteract some of these changes. However, short-term estrogen use is associated with the recurrence of vaginal bleeding (Barentsen, 1996), while long-term use has been associated with an increased risk of breast cancer (Beral, 2003), stroke (Anderson *et al*., 2004), and cardiovascular disease (Rossouw *et al*., 2002).

Phytoestrogens, including isoflavones and lignans, are estrogen like compounds naturally occurring in plant foods such as soy beans and peas, fruits, vegetables and nuts and

grains. These compounds can activate the estrogen receptor and induce messenger RNA transcription. Depending on the situation, binding to the receptor in the presence of endogenous estrogen in pre-menopausal women could result in an antagonist action by competitive binding, whereas in the postmenopausal state, phytoestrogens have been hypothesized to act as an agonist. If that is the case, they could provide an alternative for traditional estrogen therapy. The normal consumption of phytoestrogens in western population is very low, less than 5 mg/d, but the consumption in Asian population is 10- to 40-fold higher; the estimated intake of women in Shanghai is 40 mg/d. A meta-analysis on the effects of soy protein supplementation on plasma lipids comprising 38 studies reported a decrease in total cholesterol levels by 9.3% and LDL-C levels by 12.9%. Both animal and human research has suggested a preventive effect of isoflavones on bone loss and recent trials with isoflavones reported an improvement in cognitive function in both college students and postmenopausal women. Their main limitations were that the studies were small and had methodological issues, in particular the absence of blinding (Kreijkamp-Kaspers *et al*., 2004).

The extensive research on genistein (4',5,87-trihydroxy-isoflavone) over the past 10 years has occurred because of 2 sets of important observations, one resulting from investigations on the factors responsible for the lower incidence of chronic diseases in Southeast Asia and the other from the search for pharmacological agents that interfere with growth factor signaling pathways in cells. The incidence of and deaths from the hormone-dependant cancers of the breast and prostate are significantly lower in Asia than in Western populations. However, the rate of these incidences increases when Asians emigrate to Western countries (Shimizu *et al.*, 1991). A similar pattern has also been observed for cardiovascular disease. There has been an increasing interest in recent years about the dietary components in the Asian food that protect against these chronic diseases. The striking dietary difference is that Asians consume 20-50 times more soy-based food per capita than Americans do. It has been suggested that components in soybeans might be chemoprotective (Messina and Barnes, 1991). Indeed, rats maintained on a soy-based diet were significantly protected against mammary tumor induction by the carcinogens dimethylbenz[α]anthracene (DMBA) and N-methylnitrosourea (Barnes *et al.*, 1990).

Soybeans and most soy products contain large amounts of the isoflavones genistein and daidzein. Because of the structural similarity of these isoflavones to naturally occurring estrogens (Figure 2.5 and 2.6 b), it was initially suggested that these isoflavones might prevent

hormone-dependent breast- and prostate cancers by virtue of their potential estrogen-antagonist activity (Kim *et al*., 1998).

2.9 Delivery systems of probiotic organisms

For a potential therapeutic effect, probiotic organisms need to be delivered in the active form. Dairy and soy foods may serve as the ideal systems for the delivery of probiotic bacteria to the human GIT, since they may provide a favorable environment, which promotes growth and enhances viability of these microorganisms (Lourens-Hattingh and Viljoen, 2001). While various health claims have been associated with the consumption of probiotic organisms, they may in some instances be influenced by composition of a delivery matrix. In dairy and soy applications, probiotic organisms are delivered with different fermented dairy and soy products, most notable yoghurt and soy yoghurt. The nutritional value of the final product is also affected by processing factors, including temperature, duration of heat exposure, exposure to light, and storage conditions (Fox, 2003). Furthermore, some of these milk or soy constituents may be modified by microbial action during fermentation, which may affect the nutritional and physiologic value of the final product. In addition, bacterial strains used in the fermentation in conjunction with different added ingredients and fermentation conditions will also play an important role in the final nutritional composition of probiotic products.

During the bacterial fermentation, major constituents including lactose and milk proteins, soy proteins, raffinose, stachyose and other soy carbohydrates are utilized for the bacterial growth, which results in the conversion of fermentable materials into a range of products such as lactic acid, acetic acid, peptides, amino acids and different vitamins. In addition to exceptional nutritional attributes, milk and soy derived products such as fermented milk or soy contain components that possess a range of different bioactive compounds. Some of these bioactive compounds are considered functional, thus making dairy and soy products important part of functional foods and nutraceuticals.

2.10 Yoghurt and soy yoghurt manufacture and storage

The four main steps in yoghurt and soy yoghurt manufacture are milk preparation, heat treatment, inoculation and incubation and treatment and handling after incubation (cooling and storage).

2.10.1 Milk preparation and heat treatment

Milk is prepared by mixing 12% (w/w) total solids using low heat skim milk powder (SMP) 34% protein (Murray Goulbourn Co-operative Co. Ltd., Brunswick, Australia) in deionised water. For reconstitution, deionised water is heated to 30-40^{o}C prior to addition of SMP, followed by heating the mix to 50^{o}C with constant stirring for 30 min to dissolve solid particles. By employing a similar procedure, soymilk is prepared by mixing 4% soy protein isolate (SPI) in deionised water. For bovine yoghurt or soy yoghurt manufacturing, heat treatment of milk or soymilk is usually carried out at 85^{o}C for 30 min or 90^{o}C for 10 min for milk and 95 - 100^{o}C for 30 min for soymilk (Liu, 1997). The level of heat treatment is more severe than that used for pasteurization. There are several benefits of using such a high level of heat treatment including:

1. destruction of all pathogenic and most of spoilage bacteria,
2. inactivation of most enzymes which may cause undesirable effects to the finished product,
3. expulsion of toxic compounds and a decrease in the oxidation-reduction potential of the medium suitable for the growth of starter cultures by removal of oxygen,
4. improvement in acid production and in the quality of soymilk as a substrate (Liu, 1997),
5. conversion of calcium into a soluble form leading to a decrease in time for milk coagulation,
6. improvement in firmness and syneresis of yoghurt as a result of whey protein denaturation (mainly β-lactoglobulin and α-lactalbumin) (Lucey, 2002, 2004).

For the purpose of this review, starter culture bacteria will include yoghurt culture (*Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*) and probiotic organisms (*L. acidophilus, Bifidobacterium* sp. and *L. casei*). These organisms are typically

characterized as Gram-positive, nonspore-forming, nonmotile, catalase-negative bacteria that grow under microaerobic or strictly anaerobic conditions. The basic process of yoghurt and soy yoghurt production is outlined in Figure 2.7. During the production of yoghurt, biotransformation of milk/soymilk constituents is attributed to the fermentative action of the starter cultures, the secretion of nutritional and chemical substances by the microorganisms, as well as the presence of the microorganisms and their associated enzymes. The most dominant biochemical reaction involved during the manufacture of yoghurt is the fermentation of lactose to mainly L(-)lactic acid by LAB (Thomas and Crow, 1984). Such fermentation induced

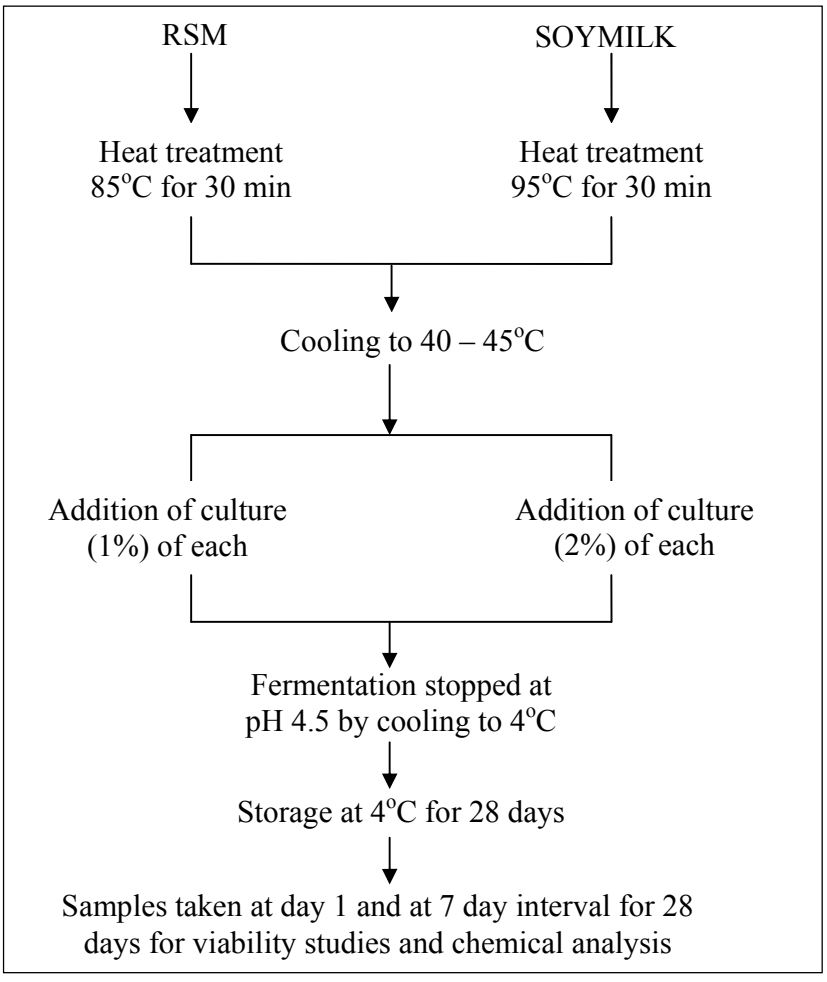

Figure 2.7 Flow diagram for the preparation of yoghurt and soy yoghurt.

acidification leads to a reduction in the pH of milk close to isoelectric point of casein resulting in the formation of smooth gel. LAB also ferment raffinose and stachyose in soymilk as well as oligosaccharides such as inulin and Hi-maize.

Numerous other changes take place concurrently, namely dissolution of colloidal calcium phosphate, hydrolysis of proteins to peptides and free amino acids, degradation of citrate to acetaldehyde and diacetyl. The total amino acid content of fermented milk or yoghurt does not differ substantially from that of milk but the free amino acid content is higher due to proteolytic activity of LAB (Rasic and Kurmann, 1983).

2.10.2 Inoculation and incubation

The heat treated milk/soymilk is cooled to 42^{o}C prior to inoculation. The level of inoculum of starter cultures can range from 1 to 2%. Although yoghurt/soy yoghurt can be produced in a short time due to rapid acid production when inoculated with a high level of starter culture (5%), this leads to defects in aroma and syneresis (Biliaderis *et al*., 1992; Lucey, 2004). The optimum level is 1% of each of *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus*. The probiotic culture can be added prior to fermentation simultaneously with the conventional yoghurt cultures or after fermentation to the cooled (4^{o}C) product before packaging. The inoculation is usually carried out at 42^{o}C. As the optimum temperatures for the growth of *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* are 37^{o}C and 45^{o}C, respectively, the incubation at 42^{o}C would favour the growth of both microorganisms. During incubation, associative growth between the two bacteria provides suitable growth conditions for each other. For example, glycine, valine and hystidine amino acids produced as a result of casein hydrolysis by *L. delbrueckii* ssp. *bulgaricus* have been shown to promote the growth of *S. thermophilus* (Tamime and Robinson, 1985; Shah, 2003), whereas the latter produces formic acid, which stimulates the growth of the former. Moreover, *S. thermophilus* starts to grow in the initial phase of the incubation producing acid and depleting oxygen from milk, thus making oxidation-reduction potential more favorable for *L. delbrueckii* ssp. *bulgaricus*. As the pH of milk drops to 5.40, *L. delbrueckii* ssp. *bulgaricus*, which is acid-tolerant, continues to grow and produces lactic acid until a desire pH in the product is reached. For a set yoghurt, the incubation is carried out in tubs, while for a stirred yoghurt it is carried out in bulk and the gel is then broken to provide a smooth and homogeneous gel. The end point of incubation may depend on the food standard of each country. The Australian Food Standard Code requires that the pH value of yoghurts be no more than 4.50, while the US standard requires a minimum tritratable acidity of 0.90%.

2.10.3. Treatment and handling after incubation

Once the desired level of acidity has been achieved, the yoghurt/soy yoghurt is cooled and stored at 4^{o}C. During the first 24 h of storage, a distinct change occurs in the set yoghurt. The level of syneresis decreases as a result of the hydration of casein micelles (Shah, 2003; Lucey, 2004). In addition, the gel strength also increases as the temperature of the set yoghurt is decreased (Guyomarc'h *et al*., 2003). Therefore, it is very important to store the product overnight at 4^{o}C and avoid rough mechanical handling to prevent excessive syneresis.

In recent years some yoghurt products have been reformulated to include live *L. acidophilus* and *Bifidobacterium* (known as AB-cultures) in addition to the conventional yoghurt starter microorganisms, *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* (Lourens-Hattingh and Viljoen, 2001). In Australia and Europe, yoghurt containing *L. acidophilus* and *Bifidobacterium* sp. is referred to as AB yoghurt. The recent trend is to incorporate *L. casei* in addition to *L. acidophilus* and *Bifidobacterium* sp., and such products are known as 'ABC yoghurt' (Shah, 2003).

2.10.4. Quality of soy yoghurt

The quality of yoghurt/soy yoghurt can be assessed through its chemical, microbiological and physical properties. In general, the chemical and microbiological parameters are controlled under food legislation of each country. For example, the Australian Food Standard Code (Standard 2.5.3, 2004) requires that the viable counts of yoghurt starter cultures be no less than 10^6 cfu/g of a product throughout the storage period. The protein content must be no less than 30 g/kg while the pH of product must be less than 4.50. In some European countries, the use of stabilizers is prohibited (De Vyust and Degeest, 1999). However, there is no legal requirement for the physical properties of a product. In general, set yoghurt should be firm, smooth in texture, free from lump or graininess and spoonable without any syneresis on the surface of the product (Tamime and Robinson, 1999). Several methods including sensory and instrumental assessments have been used for assessing physical properties of yoghurts.

2.11 Sensory evaluation

Sensory evaluation is defined as the science of judging and evaluating the quality of a food by the use of the senses, i.e. taste, smell, sight, touch and hearing. Sensory testing has been developed into a precise, formal, structured methodology that is continually being updated to refine existing techniques. The developed methods serve economic interests and can establish the worth or acceptance of a commodity. Before a product reaches the market, it has gone through many tests to accurately judge how well the public will accept it. This is especially true in the food industry because of the many taste and social preferences (Meilgaard *et al*., 1991). There are many questions that need to be answered before a company is willing to risk hundreds of thousands of dollars producing, transporting, and marketing a new product. Some possible questions include: (i) Will anyone like the product? If so, Who? (ii) Would anyone of those people be willing to buy the product? If so, at what price? (iii) How can the product be successfully marketed to those people? and (iv) Will anyone prefer the product over another to capture some of the market share of that food category? If so, how much? (Bopp, 1997).

In today's business world, nothing is left to chance. Testing the product before making a serious investment is a simple and effective way to answer these questions with relative certainty. Of course, nothing is ever guaranteed, but consumer testing is the best way to "test the water" for a new product. Consumer testing by affective testing is a useful tool employed to try to answer questions about the success of a new product. Although there are many different types of consumer tests, the affective test is the most popular for basic consumer tasting of food. Affective tests, when done properly:

- Allow different treatments to be judged to find the optimum accepted product.
- Break the masses of consumers down into smaller groups to allow an understanding of who will buy the product and how to market it to them.
- Assess the market share potential for the new product. In addition, other products can be improved upon by testing results.

Information is obtained by asking specific questions about a person’s age, sex, geographic location, nationality, religion, education and employment along with their preferences on the product being tested. To put it more simply, it stereotypes user groups based on these variables and learns the preferences of particular groups' eating habits. This is not done because of

prejudicial motivation, but simply due to consumer preferences which tend to be very grouped, based on such factors listed above. This type of testing is a very accurate tool in understanding consumer preferences (Bopp, 1997).

Sensory tests offer a course to select a product that optimizes value for money. Sensory evaluation is used as a practical application in product development by aiding in product matching, improvements, and grading. Research is another area where sensory evaluation is frequently used. Evaluation of a product may be needed to determine the effects an experiment had on its subject. Finally, quality control and marketing is yet another application of sensory testing (Stone and Sidel, 2004; Meilgaard *et al*, 1991).

Simply stated, sensory evaluation is divided into two methods, subjective and objective testing. Subjective tests involve objective panelists, while objective testing employs the use of laboratory instruments with no involvement of the senses. Both tests are essential in sensory evaluation and necessary in a variety of conditions (Meilgaard *et al*., 1991).

2.11.1 Hedonic scale

One such subjective test is the use of the hedonic scale method (Figure 2.8). This rating scale method measures the level of the liking of foods, or any other product where an affective tone is necessary. This test relies on people's ability to communicate their feelings of like or dislike. Hedonic testing is popular because it may be used with untrained people as well as with experienced panel members. A minimum amount of verbal ability is necessary for reliable results (Poste *et al*., 1991; O'Mahony, 1986).

In hedonic testing, samples are presented in succession and the subject is told to decide how much he likes or dislikes the product and to mark the scales accordingly. The nature of this test is its relative simplicity. The hedonic scale is anchored verbally with nine different categories ranging from like extremely to dislike extremely. These phrases are placed on a line-graphic scale either horizontally or vertically. Many different forms of the scale may be used with success, however, variations in the scale form is likely to cause marked changes in the distribution of responses and ultimately in statistical parameters such as means and variances (ASTM, 1968). Hedonic ratings are converted to scores and treated by rank analysis or analysis of variance. Hedonic scales are used with both experts and untrained consumers, with the best

Name: _____ Product code: _____Date:_____

Please tick the term that best describes your attitude about the product.

____Like extremely
____Like very much
____Like moderately
____Like slightly
____Neither like or dislike
____Dislike slightly
____Dislike moderately
____Dislike very much
____Dislike extremely
Comments:

Figure 2.8 An example of the nine-point hedonic scale.

results obtained with an untrained panel (Amerine *et al*., 1965). The ratings obtained on a hedonic scale may be affected by many factors other than the quality of the test samples. Characteristics of the subjects, the test situation, attitudes or expectations of the subjects can all have a profound effect on results. A researcher needs to be cautious about making inferences on the bases of comparison of average ratings obtained in different experiments (Stone and Sidel, 2004; Poste *et al*., 1991; ATSM, 1968).

2.11.2 Facial scales

These scales were primarily intended for use with children and those with limited reading and /or comprehension skills. They can be described as a series of line drawings of facial expressions ordered in a sequence from a smile to a frown, or they may depict a popular cartoon character as shown in Figure 2.9. The facial expression may be accompanied by a descriptive phrase and may have five, seven or nine categories. For computational purposes,

these facial expressions are converted to their numerical counterparts and treated statistically (Stone and Sidel, 2004).

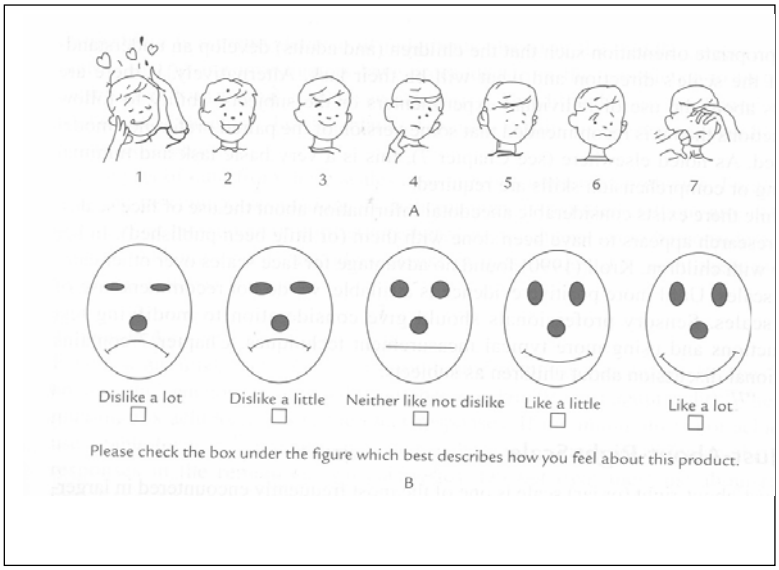

Figure 2.9 Two examples of face scales that can be found in the literature and appear to have been used for measuring a child's responses to products (Stone and Sidel, 2004).

2.11.3 Other sensory tests

There are a number of different sensory tests as indicated by the following series of definitions besides hedonic and face scales used in the sensory evaluation of a food product.

Difference tests: In difference tests the panelists are merely asked if a difference exists between two or more samples.

Preference tests: Preference or acceptance tests determine representative population preferences and these tests inherently require many people on the panel.

Triangle test: In the triangle test, three coded samples are presented to the panelist. He/she is told that two samples are identical and he/she is asked to indicate the odd one.

Duo-Trip test: In the duo-trio test, three samples are presented to the taster. One is labeled "R"(reference) and the other two are coded. One coded sample is identical with "R" and the other is different. The panelist is asked to identify the odd sample.

Paired comparison test: In the paired comparison test, a pair of coded samples that represent the standard or control and an experimental treatment are presented to the panelist,

who is asked to indicate which sample has the greater or lesser degree of intensity of a specified characteristic, such as sweetness and hardness. If more than two treatments are being considered, each treatment is compared with every other in the series.

Multiple comparison: In multiple comparison tests, a known reference or standard is labeled "R" and presented to the panelist with several coded samples. The panelist is asked to score the coded samples in comparison with the reference sample.

Ranking: The panelist is asked to rank several coded samples according to the intensity of some particular characteristic.

Scoring: Coded samples are evaluated by the panelist who records his reactions on a descriptive graduated scale. These scores are given numerical values by the person who analyzes the results.

Flavor-profile method: The flavor-profile method consists of a small laboratory panel of 6 or 8 people trained in the method measure of the flavor profile of food products. Descriptive words and numbers, with identifiable meaning to each panel member, are used to show the relative strength of each note on a suitable scale.

Dilution tests: Dilution tests involve the determination of the identification threshold for the material under study. Determining the type of research that is being done, and the type of evaluation that is needed is crucial in obtaining accurate results from a sensory project (Poste *et al*., 1991; Sensory Evaluation Introduction, 1997; Stone and Sidel, 2004).

2.12 Rheology

"Rheology is the science of the deformation and flow of materials" (Prentice, 1992; Steffe, 1992). This is particularly important for foods because they have to be deformed before ingestion. Many phenomena occur during eating. One of those is mouthfeel that can be defined as the sensory experience derived from the sensation in the mouth and on the tongue after ingestion of food (McKenna, 2003). Mouthfeel is influenced by the textural and rheological characteristics of foods.

2.12.1 Measurement of rheological and textural characteristics of yoghurt and soy yoghurt

There are several testing procedures and instruments available for assessing textural and rheological characteristics of yoghurts/soy yoghurts. The testing procedures can be divided into two categories: 1) small deformation or nondestructive and 2) large deformation or destructive testing procedures. It is important to note that the nondestructive measurement cannot be used to observe mechanical characteristics of yoghurts such as fracture and yield stress since it provides only limited information on the rheological properties of the gel (Lucey, 2002; van Vliet and Luyten, 1995). Modern instruments can perform both types of measurements within the same machine. Two commonly used equipments are compression type (Instron Universal Testing Machine, Texture Analyser) and rotational type (Brookfield viscometer and Bohlin, Haake or Physica rheometer).

2.12.2 Rheological and textural properties of yoghurt and soy yoghurt

In many disciplines of science and technology, including geology and mining (Cristescu, 1989), construction technology (Tattersall and Banfill, 1983) and plastic processing (Dealy and Wissburn, 1990), rheological tests are used. However, compared with other disciplines of science and technology, the food industry is the area in which rheological tests are most extensively employed. Since milk or soymilk is able to be converted into many milk- or soymilk-based products with different physical properties, rheological studies in the food industry are of special importance. Understanding the rheological behaviour of milk or soymilk products is important in the following steps during manufacturing: (i) quality control of ingredients and finished products, (ii) design and evaluation of processing equipment, unit operations and process parameters, (iii) adjustment of time x temperature x flow rate selection of fluid dairy/soy products and (iv) characterization and development of dairy/soy products for consumer acceptability, and elucidation of the structure and relationship among structure and textural properties (Kokini, 1992; Barbaros, 2004).

2.12.3 Flow behaviour

In the evaluation of the rheological properties of a viscoelastic gel, it is necessary to define both elastic and viscous characters at the same time. Yoghurt/soy yoghurt is a semi-solid fermented milk/soymilk gel product with a firm (set) and viscous (stirred) texture, and possesses a viscoelastic property (Lucey, 2002; Puvanenthiran *et al*., 2002). The term "viscoelastic" describes materials that exhibit both the viscous property of a liquid and the elastic property of a solid simultaneously under applied stress. It also behaves as a non-Newtonian fluid with a yield value and exhibits thixotropy (decrease in viscosity with time during shearing at a specified shear rate) with partial recovery and shear-thinning behaviour (decrease in viscosity with increased shear rate) when measured under non-linear viscoelastic region (Ramaswamy and Basak, 1991; Steffe, 1992). A non-Newtonian fluid is defined as a fluid exhibiting uniform flow, but where the relation between shear stress and rate of shear is not constant, i.e. the viscosity is not constant. A minimum shear stress known as the 'yield' must be exceeded before flow begins. This type of flow known as plastic flow is often found in foods. Typical examples of this type of flow are tomato catsup, mayonnaise, whipped cream, whipped egg white margarine. The term 'plastic' refers to materials that exhibit yield stress; it does not refer to synthetic plastics (Bourne, 2002).

From the processing point of view, it means that an inappropriate use of processing and mechanical handling can cause breakage of a set gel, which will never fully recover or may decrease the apparent viscosity of a stirred product influencing the quality of the product. Similarly, distribution of stirred yoghurt as soon as it is packed can result in a product of low viscosity. Apparent viscosity is defined as the viscosity of a non-Newtonian fluid. Non-Newtonian liquids are usually complex and consist of more than one phase, although polymer solutions may be treated as a single phase. There is always a continuous phase and one or more dispersed phases (Muller, 1973; Bourne, 2002).

Qualitatively, the rheology of a dispersed system depends on the properties of the continuous phase, the dispersed phase, and the interaction between the two. In the continuous phase, viscosity, chemical composition, pH, and electrolyte concentration are of importance. In the dispersed phase, which may be liquid or solid, i.e. emulsion and suspension respectively, volume concentration (percentage of one in the other), the viscosity (if an emulsion), particle size, shape, size distribution, and chemical composition play a role. The interaction between the two phases may be affected additionally by stabilizing and surface active agents, and the

properties of any stabilizing film may modify the behaviour. Perhaps one can visualize a thinning system as a fluid where either chemical bonds are broken or where the particles align with increasing shear rate. Thus viscous friction decreases with increasing shear rate. In time dependence the *status quo* is reached slowly; in steady state phenomena, rapidly. With thickening systems electrical forces have been suggested which are stronger at higher shear rates (Muller, 1973).

Simple liquids and true solutions are usually Newtonian i.e. a true viscous flow. Examples of liquid foods exhibiting Newtonian behaviour are tea, coffee, beer, and carbonated beverages, alcoholic drinks, meat extracts, corn syrup, sucrose and salt solutions, maple syrup, some light cooking oils, some honeys, milk, dilute solutions of gum arabic, and water. The shear rate (γ) is directly proportional to the shear stress (τ) and the viscosity is independent of the shear rate within the laminar flow range. Thus a Newtonian liquid is a liquid for which a graph of shear stress (τ) against shear rate (γ) is a straight line (Figure 2.10a).

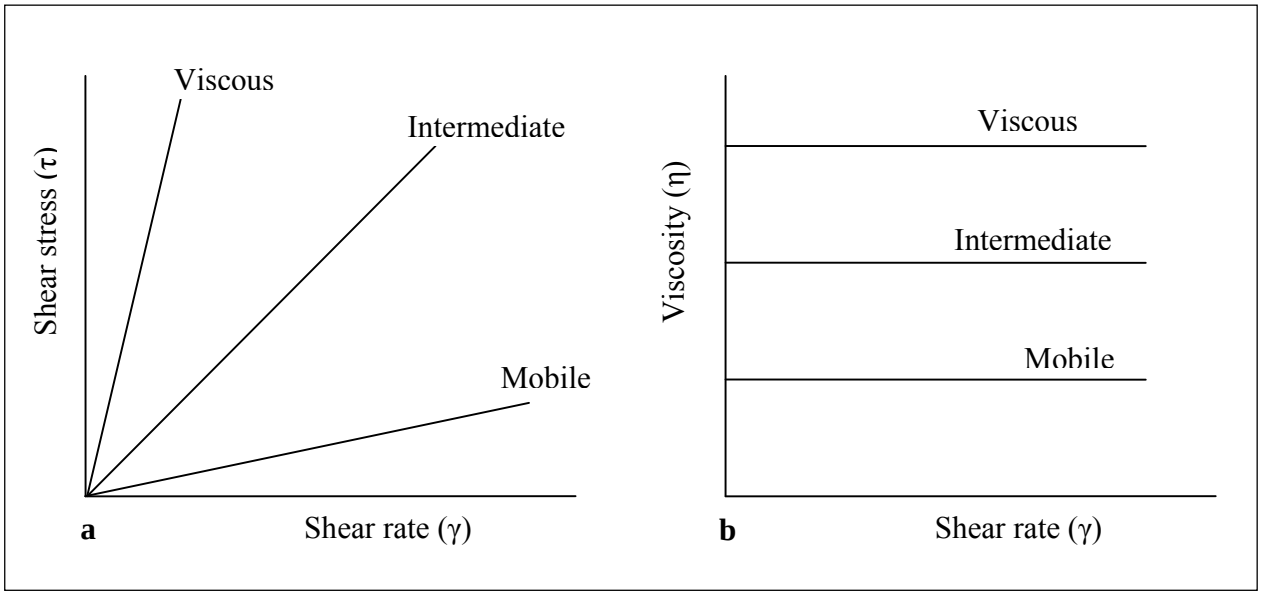

Figure 2.10 Newtonian flow: (a) shear stress versus shear rate; (b) viscosity versus shear rate (the viscosity remains constant with changing shear rate).

A Newtonian fluid possesses the simplest type of flow properties. The characteristics of this type of flow are described by the equation given below:

$\tau = \eta \gamma$

where η is the coefficient of viscosity (Pa s), τ is the shear stress (Pa), γ is the shear rate (s^{-1}). The proportionality constant is called the coefficient of viscosity and is often referred to as the 'dynamic viscosity' and is very dependent on temperature. A fluid with high viscosity is called 'viscous' whereas a fluid with low viscosity is called 'mobile (Muller, 1973; Bourne, 2002).

2.12.4 Types of flow behaviour

Another type of plastic flow is the type in which the shear stress-shear rate plot is nonlinear above the yield stress. The curve may be concave downward (dilatant with a yield stress), or convex downward (pseudoplastic with a yield stress). It is sometimes known as the 'mixed type'.

Table 2.2 Four types of flow behaviour

Time independent (steady state)	***Time dependent***
Pseudoplastic	Thixotropy
Dilatancy	Rheopexy

2.12.4.1 Pseudoplastic

In this type of flow, an increase shear force gives a more than proportional increase in shear rate, but the curve begins at the origin. While subjected to high rates of shear (e.g. stirring) the liquid is thinner than when sheared slowly. The apparent viscosity depends on the rate of shearing but not on the length of time that shearing has proceeded. The τ-γ curve is not a straight line (Figure 2.11). The shear rate increases more rapidly in proportion to the shear stress so the apparent viscosity decreases with increasing shear rate. For every τ there is a definite γ and *vice versa*, Therefore, the phenomenon is referred to as showing 'steady state' behaviour (Muller, 1973; Bourne, 2002).

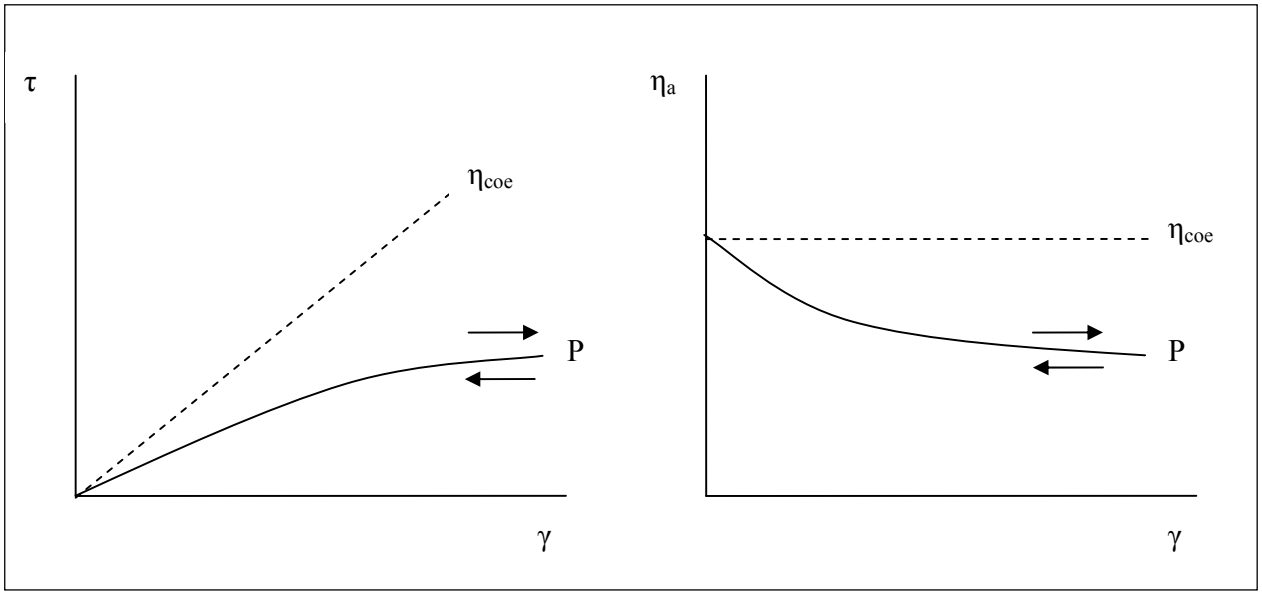

Figure 2.11 Pseudoplastic (P) flow curves. The arrows indicate increase or decrease of shear rate γ in a continuous experiment. η_{coe} = coefficient of viscosity; η_a = apparent viscosity.

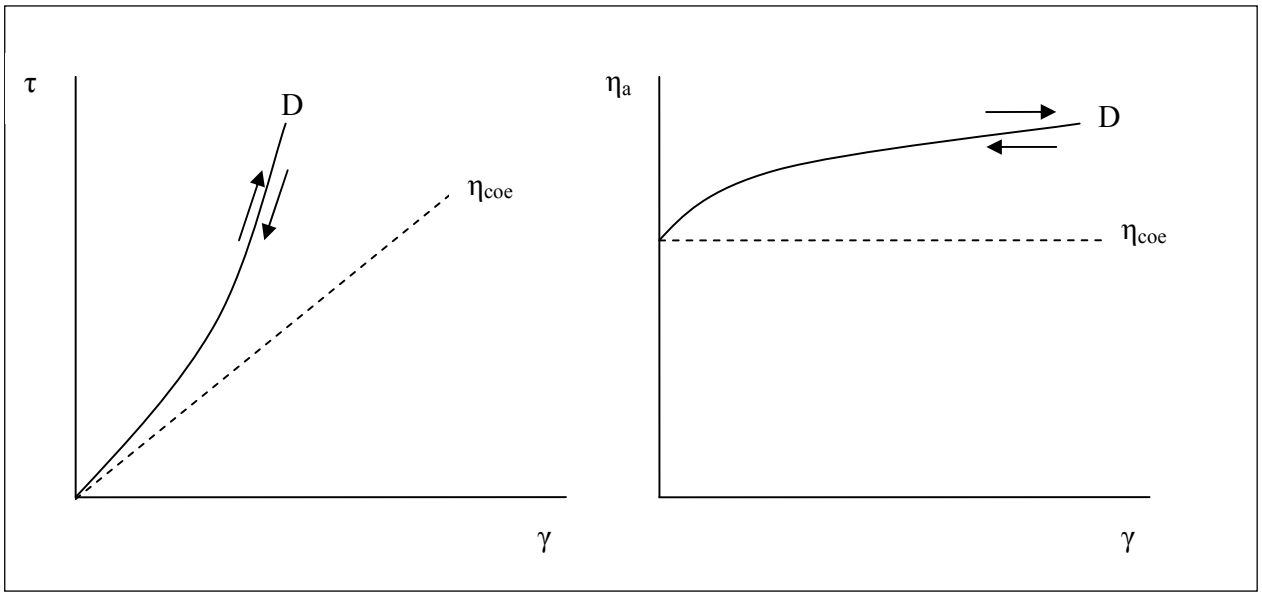

Figure 2.12 Dilatant (D) flow curves. The arrows indicate increase or decrease of shear rate γ in a continuous experiment. η_{coe} = coefficient of viscosity; η_a = apparent viscosity (Muller, 1973; Bourne, 2002).

2.12.4.2 Dilatancy

Figure 2.12 shows a time independent thickening at high rates of shear, the opposite of pseudoplasticity. The shear stress-shear rate plot begins at the origin but is characterized by equal increments in the shear stress giving less than equal increments in the shear rate. The curve is not linear but values of τ and γ are uniquely related (steady state thickening). Apparent viscosity η_a increases with increasing γ. Examples are high solids, raw starch suspensions, and some chocolate syrups. This type of flow is only found in liquids that contain a high proportion of insoluble rigid particles in suspension. Dilatant flow is fairly rare in the food industry and extremely rare in finished food products (Muller, 1973; Bourne, 2002).

2.12.4.3 Thixotropy

The apparent viscosity decreases with the time of shearing but the change is reversible; that is, the fluid will revert to its original state ('rebuild itself') on standing. Thixotropy is time dependent softening. The plot is curved and similar to pseudoplasticity, in that η_a decreases with increasing shear rate. It differs, however, in that the decrease is not only related to shear rate but also to time. With the same shear rate, the η_a decreases with time so η_a depends not only on the rate but also on the duration of shear. The τ-γ curve is plotted by increasing γ by definite intervals to a maximum and then decreasing it in the same way to zero. The time intervals between the readings and the readings themselves must be kept constant (Figure 2.13). When the rate of shear is decreased the material only slowly thickens to give its original behaviour. If the τ-γ diagram is time dependent, non-Newtonian flow results in a loop (Figures 2.13 and 2.14). This is referred to as a hysteresis loop. (The term hysteresis is also applied to delayed elastic phenomena, hence a general definition of hysteresis is: a deformation process where the loading and unloading parts do not coincide, but form a loop). An important consequence of this time dependency is that the testing routine must be strictly timed. Not only the test itself but the previous history of the sample (handling) must be controlled. For instance, sampling and filling the viscometer cup must be subjected to a definite routine. This is not necessary with time independent samples. The material is said to have a rheological memory. This condition is frequently found in food systems. Some starch paste gels are in this class (Muller, 1973; Bourne, 2002).

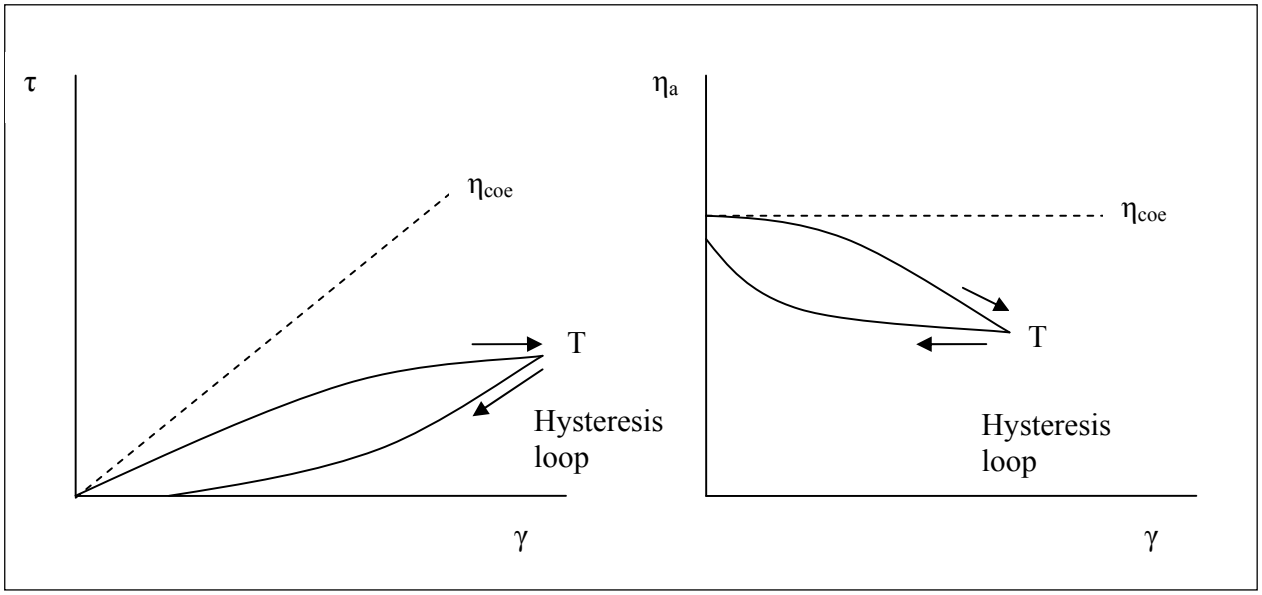

Figure 2.13 Thixotropic (T) flow curves. The arrows indicate increase or decrease of shear rate γ in a continuous experiment. η_{coe} = coefficient of viscosity; η_a = apparent viscosity (Muller, 1973; Bourne, 2002).

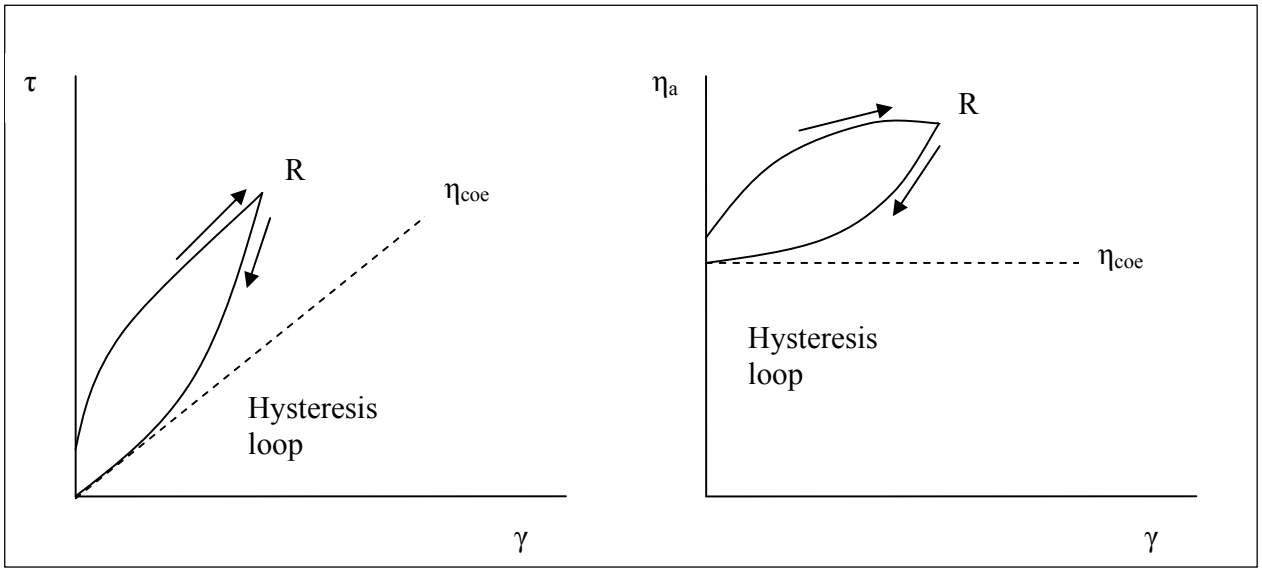

Figure 2.14 Rheopectic (R) flow curves. The arrows indicate increase or decrease of shear rate γ in a continuous experiment. η_{coe} = coefficient of viscosity; η_a = apparent viscosity(Muller, 1973; Bourne, 2002).

2.12.4.4 Rheopexy

This is time dependent thickening. The apparent viscosity increases with time of shearing and the change is reversible; that is, after resting, the product returns to its original apparent viscosity. The τ-γ relationship is curvilinear and τ and γ are not uniquely related. There are two values of γ for each τ and vice versa if the cyclic testing routine is followed. Rheopexy is the opposite of thixotropy and the same time-control on testing applies. It is rare to find this type of behaviour in a food system (Figure 2.14) (Muller, 1973).

2.12.5 Flow behaviour model

In pseudoplastic and dilatant systems the shear stress (τ)-shear rate (γ) relationship is not a straight line as shown in Figure 2.15. For each τ there is one corresponding value of γ therefore, the curve can be described mathematically. The model used to fit the flow behaviour data was that of Ostwald de-Waele, also known as the Power law, and is represented by the equation:

$$\eta a = \frac{\tau}{\gamma} = k\gamma n^{-1}$$

where η_a is the apparent viscosity (Pa s), τ is the shear stress (Pa), γ is the shear rate (s^{-1}), *k* is the consistency index (Pa s^n), and *n* is the flow behaviour index. The larger the value of *k*, the thicker the product and therefore more viscous is the fluid. Thus the latter is a measure of departure from Newtonian behaviour. In this model the parameter *n* constitutes a physical property that characterizes a non-Newtonian behaviour and when $n < 1$, thinning occurs and the fluid is pseudoplastic. If $n = 1$, the fluid is Newtonian and *k* = the coefficient of viscosity. If $n > 1$, thickening occurs and the fluid is dilatant (Muller, 1973; Bueno and Garcia-Cruz, 2001).

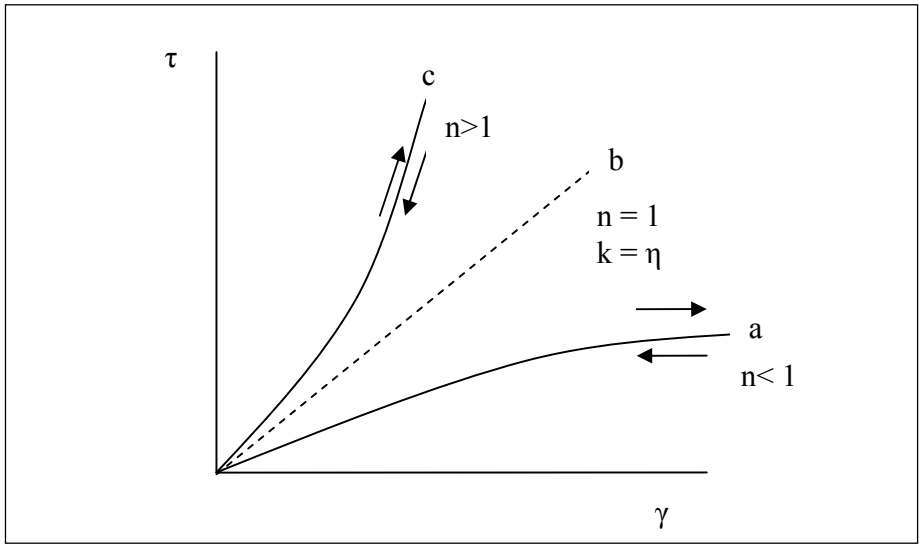

Figure 2.15 (a) Pseudoplastic, (b) Newtonian, (c) dilatant flow and *n* is the flow behaviour index. The arrows indicate increase or decrease of shear rate γ in a continuous experiment (Muller, 1973; Bourne, 2002).

2.12.6 Oscillatory measurements (non-destructive)

Many workers have used oscillatory or dynamic testing procedures to characterize the rheological properties of milk gels (Hassan *et al*., 2002, 2003; Lucey and Singh, 1997; Lucey *et al*., 1998a, b, c; Salvador and Fiszman, 1998; Anema *et al*., 2004; Haque *et al*., 2001; Guinee *et al*., 2002). It has also been applied for stirred yoghurt. The dynamic test is regarded as a nondestructive measurement, and can be used to characterize the viscoelastic properties of milk gels (both acid and rennet type gels). Some of the main parameters that are usually determined include the elastic or storage modulus (G', which is a measure of the energy stored per oscillatory cycle and usually interpreted as the strength of gels), the viscous or loss modulus (G", which is a measure of the energy dissipated per cycle), and tan δ, which is the ratio of the viscous to elastic properties. These parameters are defined as follows:

$G' = \{\tau o/\gamma o\} \cos \delta$

$G'' = \{\tau o/\gamma o\} \sin \delta$

$\text{Tan } \delta = G''/G'$

where τo is the amplitude of the shear stress, γo is the amplitude of the strain, and δ is the phase angle. Tan δ is related to the relaxation of bonds in the gel during deformation and is a useful parameter for characterizing gels (Lucey, 2002).

3.0 Effect of acidification on the activity of probiotics in yoghurt during cold storage

3.1 INTRODUCTION

Fermented dairy foods have long been considered safe and nutritious. The health benefits elicited by lactic acid bacteria (LAB) involved in the production of these foods were the primary reason for Metchnikoff to associate the consumption of yoghurt with longevity of Bulgarian peasants (Lourens-Hattingh and Viljoen, 2001). The beneficiary role of yoghurt may be further enhanced by the supplementation of *Lactobacillus* and *Bifidobacterium* sp., resulting in a product termed AB yoghurt (Shah, 2000a). *L. acidophilus*, *Bifidobacterium* sp. and *L. casei* are considered probiotic organisms since they are believed to exert beneficial health effects in the host by modulating the intestinal microflora (Schrezenmeir and De Vrese, 2001). A number of health benefits have been proposed including antimicrobial, antimutagenic, anticarcinogenic and antihypertensive properties, and reduction in serum cholesterol, alleviation of lactose intolerance, and reduction of allergic symptoms (Scheinbach, 1998; Shah, 2000a; Schrezenmeir and De Vrese, 2001; Lourens-Hattingh and Viljoen, 2001).

Besides their desired health properties, probiotics should meet several basic requirements for the development of marketable probiotic products including their survival and activity in the product, and stability during storage of the product. In addition, probiotics should not adversely affect the taste or aroma of the product nor acidification during the shelf life of the product (Heller, 2001). There is no general agreement on the minimum concentration of probiotics to achieve therapeutic benefits. While some researchers suggest concentration levels above 10^6 cfu/mL (Kurmann and Rasic, 1991), others stipulate > $10^7$and 10^8 cfu/mL as satisfactory levels (Davis *et al*., 1971; Kailasapathy and Rybka, 1997; Lourens-Hattingh and Viljoen, 2001). The Australian Food Standard Code requires that the pH of yoghurt be a minimum of 4.50 in order to prevent the growth of any pathogenic organisms (Micanel *et al*., 1997).

Numerous factors may affect the survival of *Lactobacillus* and *Bifidobacterium* sp. in yogurt. These include strains of probiotic bacteria, pH, presence of hydrogen peroxide and dissolved oxygen, concentration of metabolites such as lactic acid and acetic acids, buffering capacity of the media as well as the storage temperature (Shah and Jelen, 1990; Dave and Shah 1997; Shah, 2000b; Shah and Ravula, 2000; Talwalkar and Kailasapathy, 2004). The viability also depends on the availability of nutrients, growth promoters and

Donkor, O. N, Henriksson, A., Vasiljevic, T., & Shah, N. P. (2006). Effect of acidification on the activity of probiotics in yoghurt during cold storage. *International Dairy Journal*, *16*, 1181-89.

inhibitors, concentration of solutes (osmotic pressure), inoculation level, incubation temperature, fermentation time and storage temperature (Dave and Shah, 1997; Shah, 2000b). The main factors for loss of viability of probiotic organisms have been attributed to the decrease in the pH of the medium and accumulation of organic acids as a result of growth and fermentation (Shah and Jelen, 1990; Shah, 2000b). Among the factors, ultimate pH reached at the end of yogurt fermentation appears to be most important factor affecting the growth and viability of *Lactobacillus acidophilus* and especially *Bifidobacterium* sp. (Laroia and Martin, 1991; Shah and Ravula, 2000). Metabolic activity of *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus* during storage resulting in the production of organic acids (termed post-acidification) may further affect cell viability of probiotics (Shah and Ravula, 2000). However, there is little information on the effects of the termination pH and post-acidification on the survival and activity of probiotics.

The aims of this study were to examine the effect of different termination pH of yoghurt and of post-acidification on the activity of probiotic bacteria during refrigerated storage and to further assess the effect of proteolytic activity of the organisms on the viability of bacteria.

3.2 MATERIALS AND METHODS

3.2.1 Experimental design

This study examined the chemical and physiological changes of set yoghurts made with 12% (w/w) total solids low heat SMP 34% protein (Murray Goulbourn Co-operative Co. Ltd., Brunswick, Australia) fermented using starter cultures and probiotic cultures and terminated at different pH. Eight batches of set yoghurts were produced as shown in Table 3.1. All batches were held at 42^{o}C until the termination pH of 4.45, 4.50, 4.55 or 4.60 was reached. The parameters that were evaluated included bacteria cell counts, organic acid concentrations, pH changes, and proteolytic activity at inoculation, overnight, storage and at weekly intervals for 4 weeks during storage at 4^{o}C.

3.2.2 Propagation of cultures

Pure strains of *S. thermophilus* St1342 and *L. delbrueckii* ssp. *bulgaricus* Lb1466 were obtained from the Victoria University Culture Collection (Werribee, Australia). Strains of *L. acidophilus* LAFTI® L10, *B. lactis* LAFTI® B94 and *L. paracasei* LAFTI® L26 were kindly provided by DSM Food Specialties (Sydney, NSW, Australia). Each strain was stored at -80^{o}C. Sterile 10 mL aliquots of de Man Rogosa and Sharpe (MRS) broth (Sigma Chemical Co., St Louis, USA) were inoculated with 1% of each strain and incubated at 42^{o}C

for *L. delbrueckii* ssp. *bulgaricus* Lb1466 and 37°C for *S. thermophilus* St1342, *L. acidophilus* LAFTI® L10, *B. lactis* LAFTI® B94 and *L. paracasei* LAFTI® L26. For propagation of bifidobacteria, sterile MRS broth was supplemented with 0.05% L-cysteine.hydrochloride (Sigma) to provide anaerobic condition and stimulate their growth (Ravula and Shah, 1998). The activated organisms after three successive transfers were used for the production of cultures for yoghurt making. The cultures were prepared by inoculating 1% (v/v) in 10 mL aliquots of reconstituted skim milk (RSM) supplemented with 2% glucose and 1% yeast extract with each activated culture.

3.2.3 Yoghurt preparation and storage

The yoghurt mix (3 liters) was prepared by dissolving SMP in Milli Q water at 120 g/L and heat treating at 85°C for 30 min followed by cooling (45°C) and aseptically inoculating with 1% (v/v) of each *L. delbrueckii* ssp. *bulgaricus* Lb1466 and *S. thermophilus* St1342. The inoculated milk was subsequently divided into two equal portions; one portion was used as a control while the other portion was inoculated with 1% (v/v) of each probiotic cultures (*L. acidophilus* L10, *B. lactis* B94 and *L. paracasei* L26). Each portion was sub-divided into four batches (each final batch was 375 mL). All batches were held at 42°C until the required pH of 4.45, 4.50, 4.55 or 4.60 was reached, at which the fermentation was terminated. The fermentation time ranged between 3.5 to 4.00 h for probiotic yoghurt and 4.00 to 4.50 h for the control yoghurt. The pH change was monitored by a pH meter (model 8417; HANNA Instruments, Singapore). Yoghurts were immediately cooled after fermentation and stored at 4°C for 28 days. The pH of all batches of yoghurt was measured at termination of fermentation, 12 h post-fermentation, and subsequently at weekly intervals.

3.2.4 Determination of viability

The colony counts of *L. delbrueckii* ssp. *bulgaricus* Lb1466, *S. thermophilus* St1342, *L. acidophilus* L10, *B. lactis* B94 and *L. paracasei* L26 were determined as described previously (Dave and Shah, 1996; Tharmaraj and Shah, 2003). The media that were selected as suitable for the viability studies included M17 agar (Amyl Media, Dandenong, Australia) and aerobic incubation at 37°C for 48 h for St1342 (Dave and Shah, 1996) and *L. delbrueckii* ssp. *bulgaricus* Lb1466 was enumerated on reinforced clostridia agar (RCA) (Oxoid, West Heidleberg, Australia) at pH 5.3 and anaerobic incubation at 42°C for 48 h (Dave and Shah, 1996). The probiotic cultures L10, B94 and L26 were enumerated using MRS – sorbitol agar, MRS – nalidixic acid, neomycin sulphate, lithium chloride and

paromomycin sulphate (NNLP; Sigma) agar, and *L. casei* agar (Ravula and Shah 1998) respectively. The incubation was performed at 37^{o}C anaerobically for 48 h for all three cultures (Laroia and Martin, 1991; Dave and Shah, 1996; Ravula and Shah 1998; Tharmaraj and Shah, 2003).

3.2.5 Determination of organic acids

Determination of organic acids was carried out according to Shah and Ravula (2000). Briefly, 3 mL yoghurt samples were mixed with 50 µL of 15.5 M nitric and 1.0 mL of 0.01M sulfuric acids. The resulting mixture was centrifuged at 14,000 x g for 30 min using an Eppendorf 5415C centrifuge (Crown Scientific, Melbourne, Australia) for removal of proteins. The supernatant was filtered through a 0.20 µm membrane filter (Schleicher & Schuell GmbH, Dassel, Germany) into an HPLC vial. The separation of organic acids was achieved using a Varian HPLC (Varian Analytical Instruments, CA, USA) fitted with an Aminex HPX - 87H, 300 x 7.8 mm ion exchange column (Biorad Life Science Group, Hercules, USA) and a guard column maintained at 65^{o}C. The mobile phase was 0.01M H_2S0_4 with a flow rate of 0.6 mL/min. Quantification of acetic and lactic acids was performed from the standard curves obtained using solutions of pre-determined concentrations.

3.2.6 Determination of proteolytic activity

Proteolytic activities of cultures used in the production of all batches of yoghurts were assessed by measuring liberated amino acids and peptides using the o-phthaldialdehyde (OPA) method of Church *et al*. (1983), Shihata and Shah (2000) and Leclerc *et al*. (2002) with some modifications. Yoghurt samples (2.50 mL) were added to 5 mL of 0.75% trichloroacetic acid and the mixture was vacuum filtered using an Advantec # 231 filter paper (MFS. Inc., CA, USA). The permeate (150 µL) was added to 3 mL of OPA reagent and the absorbance of the solution was measured spectrophotometrically at 340 nm after 2 min at room temperature (20^{o}C). The proteolytic activity of these bacterial cultures was expressed as the free amino groups measured at 340 nm as a difference in absorbance between samples containing culture and RSM.

3.2.7 Statistical analysis

Results were analysed as a split plot in time design using General Linear Model procedure of the SAS System (SAS, 1996). The univariate ANOVA test was validated by

fulfilling Huynh-Feldt (H-F) condition (Littell *et al*., 1998). All results presented are means of three independent replicates. The level of significance was preset at p = 0.05.

3.3 RESULTS

3.3.1. Viability of probiotic bacteria during cold storage

Viable counts of *L. delbrueckii* ssp. *bulgaricus* Lb1466, *S. thermophilus* St1342, *L. acidophilus* L10, *B. lactis* B94 and *L. paracasei* L26 during cold storage at various termination pH are presented in Table 3.2. There were minimal differences in the viability of St 1342 in all batches and the viable counts in general increased slightly during storage, although non-significantly (P > 0.05). The counts of *L. delbrueckii* ssp. *bulgaricus* Lb 1466 declined from 7.00 to 6.00 log cfu/g for all the control yoghurts with the exception of pH 4.50 batches which maintained fairly consistent counts of 7.00 log cfu/g throughout the storage. However, the viability of *L. delbrueckii* ssp. *bulgaricus* Lb 1466 was enhanced significant (P < 0.05) in the presence of probiotic organisms during storage (Table 3.2). *L. acidophilus* L10 appeared to tolerate acidic conditions better than the other two probiotic organisms. It generally achieved a higher concentration (8.00 log cfu/g) than a therapeutic minimum of 6.00 log cfu/g, and, although the cell concentration declined somewhat during middle storage, the organism recovered at the end of storage reaching initial concentration. The termination pH also had no significant (P > 0.05) effect on the viability of this organism.

The concentration of *L. acidophilus* L10 ranged initially between 7.35 and 7.54 log cfu/g with a non-significant difference (P > 0.05) among batches on the first day after fermentation. The trend continued until day 21 when the counts started to decline, from 7.00 to 6.00 log cfu/g. Between day 21 and 28 of storage, there was about 83% decrease in the counts. The pH change could have been the most crucial factor for the survival of *B. lactis* B94 since the decline from day 21 to 28 of 0.147-0.230 units was significant (P < 0.05). All the batches of yoghurt showed a decrease in the number of *Bifidobacterium* irrespective of the termination pH. The stability of *B. lactis* B94 in yoghurt was found to be lower than the other probiotic bacteria during storage. Similar observations were made by Dave & Shah (1997). *L. paracasei* L26 maintained a steady and constant count within the batches and throughout the storage (Table 3.2). The viability among batches was comparatively similar and there was no significant difference (P > 0.05). The consistent decline in pH during storage did not appear to affect the survival of *L. paracasei* L26. Probiotic organisms in

general remained viable above therapeutic level of 6.00 log cfu/g despite the different termination pH.

3.3.2. Decline in pH

The decline in pH of the batches of yoghurt obtained at different termination pH during refrigerated storage is shown in Table 3.3. The decline in pH was similar for all the batches of yoghurt. The pH varied between 0.057 to 0.230 units for the probiotic batches of yoghurt and 0.043 to 0.193 units for the control batches throughout the storage. Since there was no significant difference ($P > 0.05$) in pH or decline in pH values among batches during storage, it was unlikely that these changes would have affected viability of organisms due to termination pH. In general, the lower termination pH resulted in a higher pH change during cold storage.

3.3.3. Changes in lactic and acetic acid contents

Changes in lactic acid content as determined by HPLC are shown in Figures 3.1a and 1b. The concentration of lactic acid produced in the control batches of yoghurt was not significantly ($P > 0.05$) different to that in the probiotic yoghurts from day 1 until the end of storage. There was no significant change ($P > 0.05$) in the lactic acid content in all yoghurts at day 7 and 14. However, a gradual and significant ($P < 0.05$) increase in the concentration of lactic acid was observed on the last week of storage for control and probiotic yoghurts (Figures 3.1a and 1b). Overall the increase in the concentration of lactic acid was between 16% and 23% throughout the storage for the probiotic yoghurts. The changes in the concentration of lactic acid were found to be independent of termination pH, as the trend was similar among the batches (Figures 3.1a and 1b). The changes among batches were generally not significant ($P > 0.05$) during storage; however, the organisms produced significant ($P < 0.05$) amounts of lactic acid by the end of storage period. This could probably explain the reason for the decline in numbers of *B. lactis* B94 in the product (Table 3.2). The concentration of acetic acid on the other hand remained more or less consistent and the organisms did not produce a great deal of acetic acid during storage (Figures 3.2a and 2b). The increase in the concentration of acetic acid in the control batches (Figure 3.2a) followed a similar trend to those of the probiotic batches of yoghurts. The changes were not significant ($P > 0.05$) between day 1 and day 28 of storage for both types of yoghurts. The comparison of the probiotic batches to those of the control showed that the bulk of the organic acids was produced by the yoghurt cultures, but the selected probiotic organisms (*L.*

acidophilus L10 and *L. paracasei* L26) survived better in the acidic environment with viable counts above the legal requirement of 6.00 log cfu/g.

3.3.4. Proteolytic activity

During fermentation milk proteins are hydrolysed by extracellular proteinases produced by lactic acid bacteria resulting in an increase in the amount of free amino groups as quantified by the OPA method. Figure 3.3 shows the proteolytic activities in batches of yoghurt with selected probiotic strains (*L. acidophilus* L10, *B. lactis* B94, *L. paracasei* L26) in comparison to the control batches. Since proteolysis in the control batches followed a similar but non-significant ($P > 0.05$) increasing trend, results for termination pH of 4.60, 4.55 and 4.45 are not presented. There was a significant ($P < 0.05$) increase in the amount of liberated amino acids after termination at day 1 in the probiotic yoghurts compared to the control; the increase was consistent and significant throughout the storage. The extent of proteolysis with time was significantly different ($P < 0.05$) for all batches irrespective of termination pH. There were no significant differences ($P > 0.05$) in proteolytic activities among batches with the exception of termination pH of 4.45 and 4.50, which showed a significant difference ($P < 0.02$) at day 28. It could be inferred that termination pH had no effect on proteolysis. There was a significant difference in proteolytic activity between the control yoghurt and the probiotic yoghurt ($P < 0.0001$); this is a further indication of the proteolytic activity of the probiotic organisms.

3.4 DISCUSSION

The physiological state of the probiotic organisms is of special importance when selecting a strain. Several investigations showed that bacteria in the logarithmic phase are much more susceptible to environmental stresses as compared to those in stationary phase (Heller, 2001). The termination pH may affect viability due to either one or all of the factors including initial pH, fermentation time resulting in varying temperature exposure and levels of organic acids produced at the end of fermentation and during storage. A significant ($P < 0.05$) decline in the cell concentration during storage observed in our study was not a result of different termination pH but rather affected by the levels of organic acids. Viable counts of probiotic organisms in yoghurt terminated at pH 4.50 were not significantly different ($P > 0.05$) from batches produced at other termination pH. These trends were similar to both the probiotic- and the control yoghurts. Comparison of viability between batches at termination pH and within the same batch did not show a significant change during storage. The

presence of higher levels of essential growth factors such as peptides and amino acids in the yoghurt may have promoted the growth of *L. acidophilus* L10 which showed the highest counts even in increased levels of organic acids. Rybka and Kailasapathy (1995) demonstrated that *L. acidophilus* could survive in yoghurt at sufficient levels ($>10^6$ cfu/mL) for up to 28 days. It has also been reported that *L. acidophilus* survived better than the traditional yoghurt culture organisms (*L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus*) in yoghurt under acidic conditions (Shah and Jelen, 1990; Lankaputhra and Shah, 1995). *L. acidophilus* was also more tolerant of acidic conditions than *B. bifidum*.

The significant ($P < 0.05$) decline in pH accompanied with the increased concentration of organic acids (lactic acid and acetic acid) during storage probably affected the viability of *B. lactis* B94 resulting in low cell counts. *B. lactis* B94 was more sensitive to the yoghurt environment than *L. acidophilus* L10 or *L. paracasei* L26. The acidic environment on the other hand did not cause a decline in the number of *L. paracasei* L26. The number of *B. lactis* B94 and *L. paracasei* L26 (6.00 and 7.00 log cfu/g, respectively) however might have been sustained in the product by the high released amounts of free amino acids during storage. The stability of bifidobacteria and lactobacilli in fermented milk reported in the literature is variable. A poor survival for bifidobacteria in yoghurts was reported by Klaver *et al*. (1993), and Modler and Villa-Garcia (1993). Dave and Shah (1997) also reported a rapid decline in the counts of *Bifidobacterium* sp. after manufacture of yoghurt made with commercial starter cultures. In contrast, a satisfactory viability was demonstrated by Medina and Jordano (1994) and Samona and Robinson (1994). Gilliland and Speck (1977) also suggested that yoghurt should not be considered a desirable vehicle for suspending *L. acidophilus*. However, Nighswonger *et al*. (1996) concluded that the ability of intestinal lactobacilli to survive in fermented dairy products was strain dependent.

When the lactic acid content increased, pH levels correspondingly decreased during fermentation. However, even though there was a significant decline in pH during storage, there was no significant difference ($P > 0.05$) in pH changes among batches which resulted in non-significant changes in cell counts. Akalin *et al*. (2004) also found that a slight decline in pH during storage was unlikely to affect viability. Shah (2000b) observed similar decreases in pH values during storage of commercial yoghurts containing *L. acidophilus* and *B. bifidum* without any effect on cell counts. The increase in acidification in the probiotic yoghurt was mainly due to the growth of *L. delbrueckii* ssp. *bulgaricus* Lb1466, *S. thermophilus* St1342 during refrigerated storage. Studies have shown that yoghurt bacteria (*S. thermophilus* and *L. delbruekii* ssp. *bulgaricus*) survive well in yoghurt throughout the

shelf life (Hamann and Marth, 1984; Rohm, Lechner, and Lehner, 1990; Akalin *et al*., 2004). Proteolysis by yoghurt bacteria (*L. delbrueckii* ssp. *bulgaricus* Lb1466, *S. thermophilus* St1342) and probiotic bacteria (*L. acidophilus* L10, *B. lactis* B94, *L. paracasei* L26) were assessed after fermentation and during storage (Figure 3.3). Lactic acid bacteria are characterized by their high demand for essential growth factors such as peptides and amino acids. Proteinases and peptidases constitute the primary enzymes in lactic acid bacteria responsible for proteolysis in milk proteins as a source of amino acids and nitrogen (Law and Haandrickman, 1997; Shihata and Shah, 2000). In the current study, appreciable proteolytic activity was detected in all the batches of yoghurts. Even though *L. delbrueckii* ssp. *bulgaricus* Lb1466, *S. thermophilus* St1342 were proteolytic as seen in the control, they were not as proteolytic as the probiotic bacteria (*L. acidophilus* L10, *B. lactis* B94, *L. paracasei* L26). Shihata and Shah (2000) reported that *L. delbruekii* ssp. *bulgaricus* strains were less proteolytic than *S. thermophilus* strains. They also found that yoghurt bacteria (*L. delbruekii* ssp. *bulgaricus* and *S. thermophilus*) were more proteolytic than the probiotic bacteria (*L. acidophilus* and *Bifidobacterium* sp.). In our study, the probiotic bacteria released greater amounts of amino acids (Figure 3.3), suggesting that these probiotic strains were strongly proteolytic with the amount of free NH_3 groups in the yoghurt increasing linearly during storage irrespective of termination pH. The increased proteolysis could result in the better survival of probiotic organisms.

3.5 CONCLUSIONS

Different termination pH appeared to have no effect on the viability of probiotic organisms. Survival of probiotic organisms was strain dependant. The decline in pH during storage showed increased levels of organic acids resulting in low counts of *B. lactis* B94. *L. acidophilus* L10 survived better than *B. lactis* B94 and *L. paracasei* L26 in yoghurt under acidic conditions. Presence of probiotic organisms increased proteolytic activity, which appeared to have improved the survival of *L. delbruekii* ssp. *bulgaricus* Lb1466. On the other hand, increased lactic acid production by this organism, subsequently affected the survival of *B. lactis* B94 and *L. paracasei* L26. Higher levels of essential growth factors in the form of peptides and amino acids in the yoghurts may have promoted the growth of *L. acidophilus* L10 and sustained the growth of *B. lactis* B94 and *L. paracasei* L26.

Table 3.1. Summary of eight batches of yoghurts produced using starter cultures and probiotic bacteria at different strike pH

	Control yoghurt	
Batches	Termination pH	Starter cultures
1	4.60	1% (v/v) of *S. thermophilus* St 1342 + 1% of *L. delbrueckii* ssp. *bulgaricus* Lb 1466
2	4.55	1% (v/v) of *S. thermophilus* St 1342 + 1% of *L. delbrueckii* ssp. *bulgaricus* Lb 1466
3	4.50	1% (v/v) of *S. thermophilus* St 1342 + 1% of *L. delbrueckii* ssp. *bulgaricus* Lb 1466
4	4.45	1% (v/v) of *S. thermophilus* St 1342 + 1% of *L. delbrueckii* ssp. *bulgaricus* Lb 1466
	Probiotic yoghurt	
	Termination pH	Starter cultures/Probiotic cultures
1	4.60	1% (v/v) each of *S. thermophilus* St 1342, *L. delbrueckii* ssp. *bulgaricus* Lb 1466, *L. acidophilus* L10, *B. lactis* B94 and *L. casei* L26
2	4.55	1% (v/v) each of *S. thermophilus* St 1342, *L. delbrueckii* ssp. *bulgaricus* Lb 1466, *L. acidophilus* L10, *B. lactis* B94 and *L. casei* L26
3	4.50	1% (v/v) each of *S. thermophilus* St 1342, *L. delbrueckii* ssp. *bulgaricus* Lb 1466, *L. acidophilus* L10, *B. lactis* B94 and *L. casei* L26
4	4.45	1% (v/v) each of *S. thermophilus* St 1342, *L. delbrueckii* ssp. *bulgaricus* Lb 1466, *L. acidophilus* L10, *B. lactis* B94 and *L. casei* L26

Table 3.2. Effect of termination pH on viability of yoghurt culture (*L. delbruekii* ssp. *bulgaricus* Lb1466, and *S. thermophilus* St1342) and probiotic organisms (*L. acidophilus* L10, *B. lactis* B94 and *L. paracasei* L26) in yoghurts/samples

Culture	Termination pH	Period of storage, day				
		1	7	14	21	28
		Log cfu/g				
Control batches						
Lb1466	4.60*	7.45 ^{aA}	6.52 ^{bA}	7.41 ^{aA}	7.07 ^{abA}	6.54 ^{bA}
	4.55	7.77 ^{aA}	6.94 ^{bA}	7.89 ^{aA}	6.46 ^{bA}	6.94 ^{bA}
	4.50	7.70 ^{aA}	7.09 ^{abA}	7.59 ^{abA}	7.08 ^{abA}	7.02 ^{bA}
	4.45	7.92 ^{aA}	7.33 ^{abA}	6.48 ^{cA}	6.57 ^{cA}	6.66 ^{bcA}
St1342	4.60	9.14 ^{aA}	9.08 ^{aA}	9.09 ^{aA}	9.04 ^{aA}	9.22 ^{aA}
	4.55	9.18 ^{aA}	9.10 ^{aA}	9.04 ^{aA}	9.04 ^{aA}	9.20 ^{aA}
	4.50	9.16 ^{aA}	9.13 ^{aA}	9.08 ^{aA}	9.11 ^{aA}	9.23 ^{aA}
	4.45	9.06 ^{aA}	9.08 ^{aA}	9.13 ^{aA}	9.36 ^{aA}	9.14 ^{aA}
Probiotic batches						
Lb1466	4.60	7.86 ^{aA}	7.71 ^{aB}	8.19 ^{aB}	8.26 ^{aB}	8.01 ^{aB}
	4.55	7.90 ^{aA}	7.71 ^{aB}	8.18 ^{aA}	8.12 ^{aB}	8.00 ^{aB}
	4.50	7.93 ^{aA}	7.90 ^{aB}	8.33 ^{aB}	8.56 ^{aB}	8.21 ^{aB}
	4.45	7.93 ^{aA}	7.95 ^{aA}	8.08 ^{abB}	8.65 ^{bB}	8.02 ^{abB}
St1342	4.60	8.39 ^{aB}	8.39 ^{aB}	8.50 ^{aA}	8.65 ^{aA}	8.37 ^{aB}
	4.55	8.43 ^{aB}	8.38 ^{aB}	8.64 ^{aA}	8.57 ^{aA}	8.48 ^{aB}
	4.50	8.47 ^{aB}	8.36 ^{aB}	8.65 ^{aA}	8.46 ^{aA}	8.52 ^{aB}
	4.45	8.41 ^{aA}	8.43 ^{aA}	8.85 ^{aA}	8.42 ^{aB}	8.48 ^{aA}
L10	4.60	8.34 ^{a}	7.69 ^{ab}	7.68 ^{ab}	7.24 ^{b}	8.17 ^{ab}
	4.55	8.53 ^{a}	7.60 ^{b}	7.89 ^{ab}	7.21 ^{b}	8.16 ^{a}
	4.50	8.55 ^{a}	7.99 ^{ab}	7.90 ^{ab}	7.70 ^{b}	8.35 ^{a}
	4.45	8.32 ^{a}	8.02 ^{ab}	7.85 ^{ab}	7.57 ^{b}	8.32 ^{a}
B94	4.60	7.39 ^{a}	7.48 ^{a}	7.65 ^{a}	6.92 ^{ab}	6.62 ^{b}
	4.55	7.54 ^{a}	7.35 ^{a}	7.39 ^{a}	7.11 ^{a}	6.41 ^{a}
	4.50	7.41 ^{a}	7.37 ^{a}	7.37 ^{a}	7.40 ^{a}	6.93 ^{a}
	4.45	7.35 ^{a}	7.58 ^{a}	7.39 ^{a}	7.24 ^{a}	6.94 ^{a}
L26	4.60	7.72 ^{a}	7.76 ^{a}	7.63 ^{a}	7.55 ^{a}	7.67 ^{a}
	4.55	7.47 ^{a}	7.74 ^{a}	7.68 ^{a}	7.38 ^{a}	7.72 ^{a}
	4.50	7.49 ^{a}	7.80 ^{a}	7.75 ^{a}	7.73 ^{a}	7.75 ^{a}
	4.45	7.48 ^{a}	7.65 ^{a}	7.80 ^{a}	7.41 ^{a}	7.77 ^{a}
SEM				0.24		

Results presented as a mean (n = 3) ± pooled standard error of the mean (0.243). *No statistical difference among termination pH. General linear model detected no statistical difference (P = 0.2941) among means as affected by termination pH. Different small letter superscripts depict the statistical difference within a row, P < 0.05. Different capital letter superscripts depict the statistical difference (P < 0.05) between means for the same strain at the same termination pH and time.

Table 3.3. Decline in pH in batches of yoghurt produced at different termination pH during cold storage for 28 days

Culture	Time, day	Termination pH			
		4.45	4.50	4.55	4.60
Control batches	7	0.053 ^{aA}	0.060 ^{aA}	0.060 ^{aA}	0.057 ^{aA}
	14	0.055 ^{aA}	0.063 ^{abA}	0.073 ^{aA}	0.057 ^{aA}
	21	0.127 ^{abA}	0.107 ^{abA}	0.117 ^{aA}	0.070 ^{aA}
	28	0.193 ^{bA}	0.163 ^{bA}	0.163 ^{aA}	0.110 ^{aA}
Probiotic batches	7	0.077 ^{aA}	0.057 ^{aA}	0.060 ^{aA}	0.060 ^{aA}
	14	0.087 aA	0.083 ^{abA}	0.080 ^{aA}	0.077 ^{abA}
	21	0.147 ^{abA}	0.140 ^{abA}	0.110 ^{aA}	0.140 ^{abA}
	28	0.230 ^{bA}	0.183 ^{bA}	0.153 ^{aA}	0.170 ^{bA}
SEM			0.04		

Control batches – yoghurt containing yoghurt culture only; Probiotic batches – yoghurt containing yoghurt and probiotic cultures; Results presented as a mean (n = 3) ± pooled standard error of the mean (0.036). No statistical difference detected among means as affected by termination pH, P = 0.4785. Different small letter superscripts depict the statistical difference (P < 0.05) for a given block (control, probiotic); Difference between block means for a particular termination pH and time.

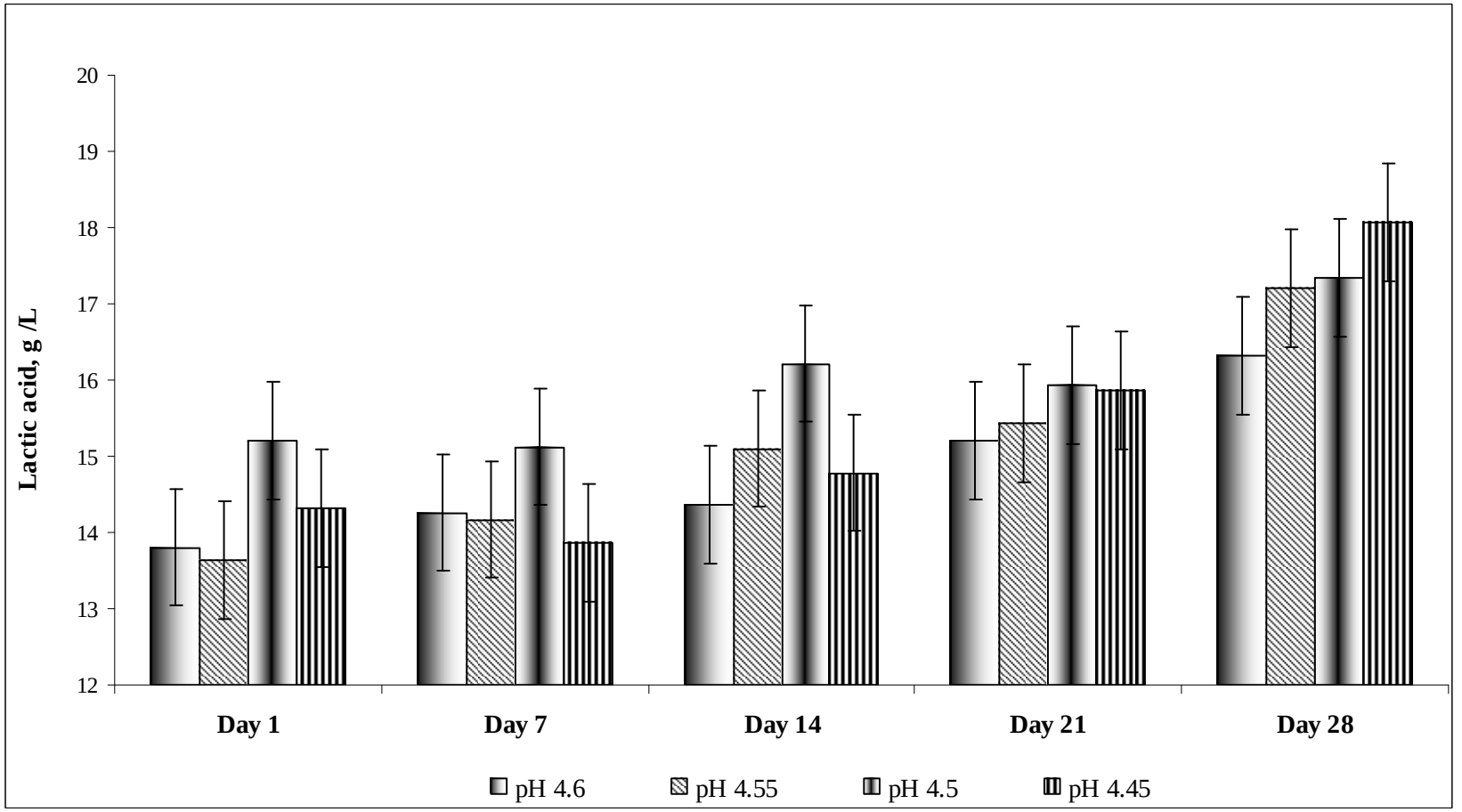

Figure 3.1a Production of lactic acid by yoghurt culture during refrigerated storage of control yoghurts produced at different termination pH 4.45, 4.50, 4.55 and 4.60. Error bars present a pooled standard error of the mean (SEM = 0.768).

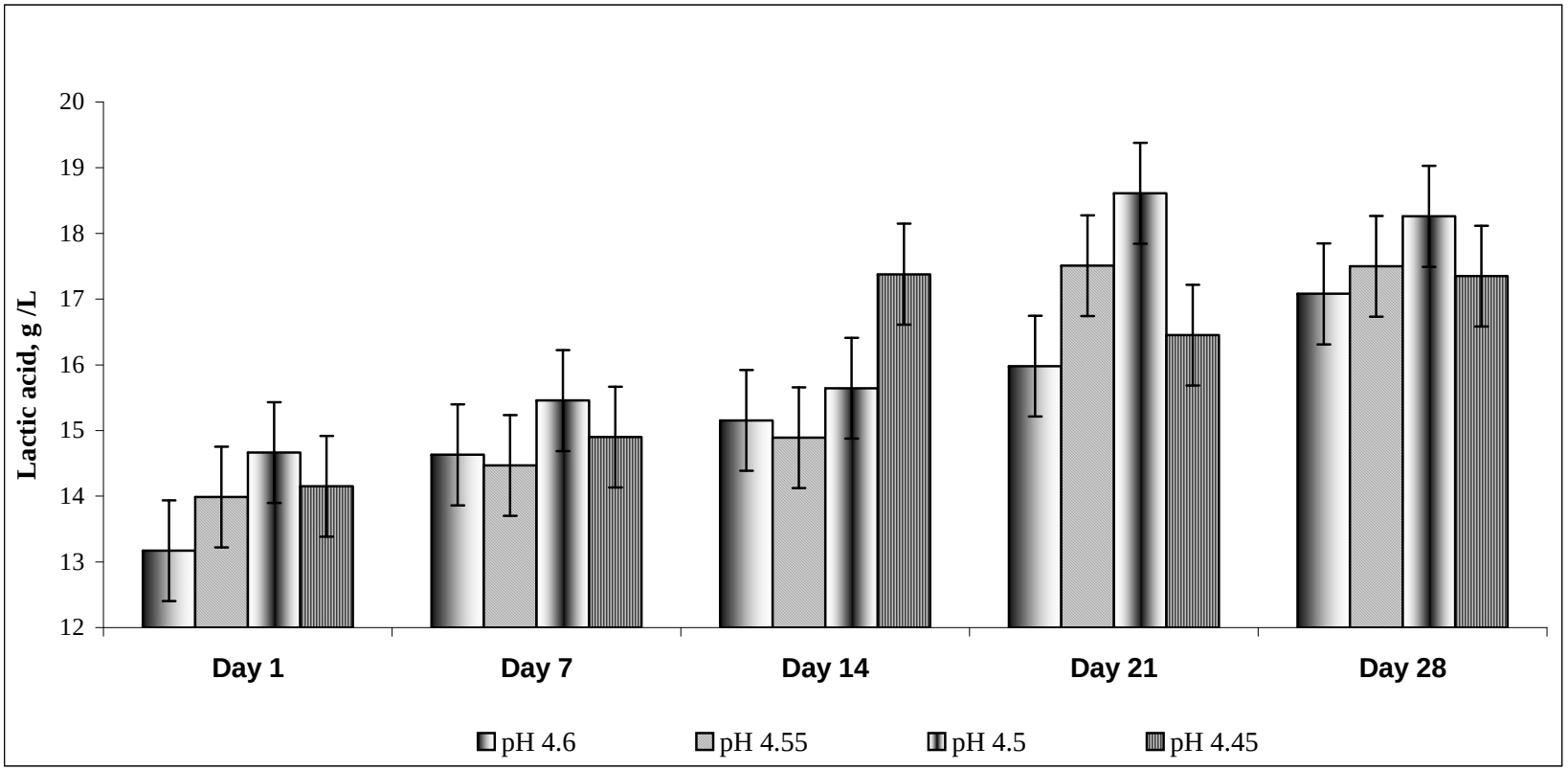

Figure 3.1b Production of lactic acid by yoghurt and probiotic cultures during refrigerated storage of probiotics yoghurts produced at different termination pH 4.45, 4.50, 4.55 and 4.60 Error bars present a pooled standard error of the mean (SEM = 0.768).

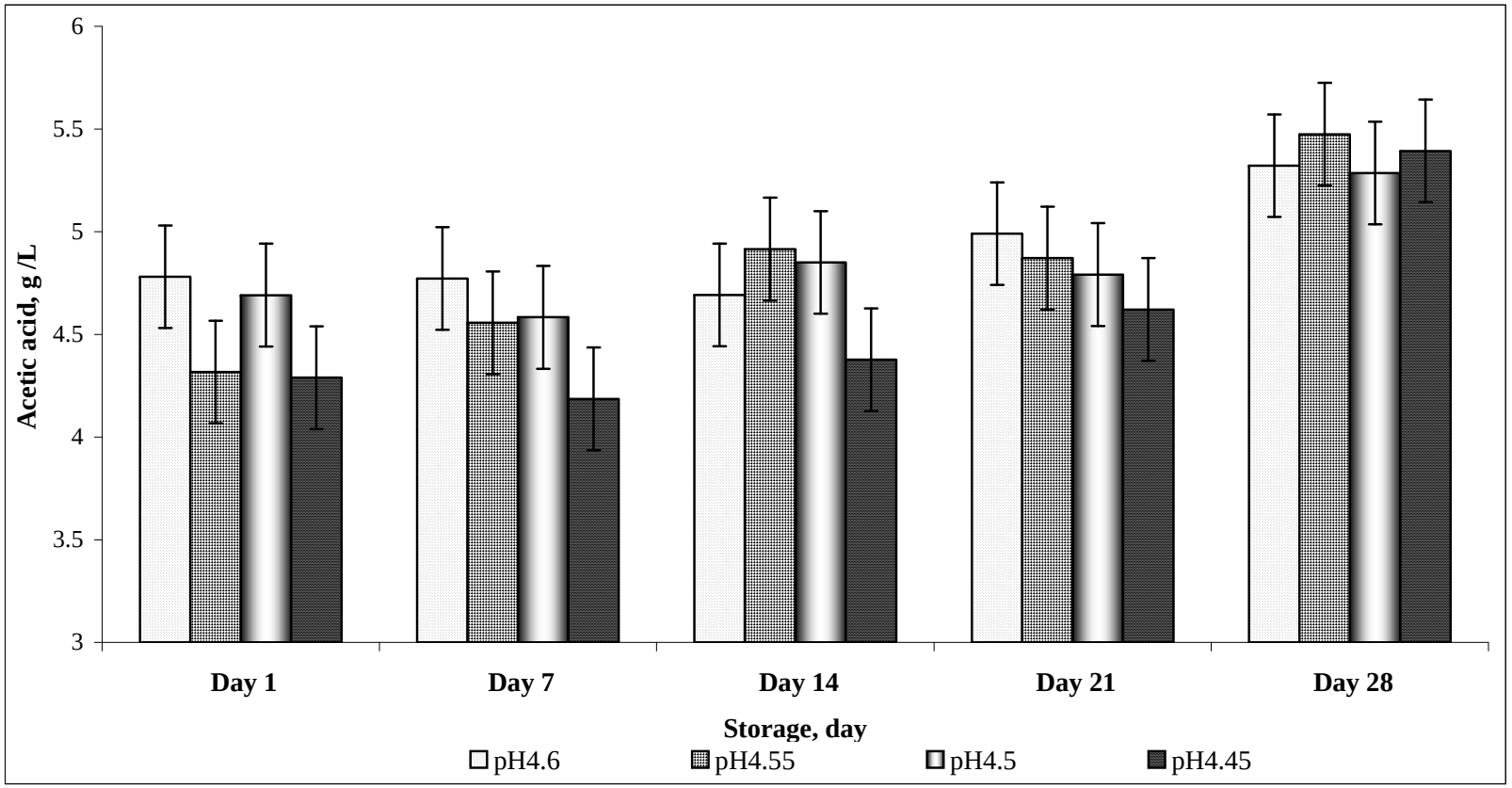

Figure 3.2a Production of acetic acid in control yoghurt by yoghurt culture during refrigerated storage at different termination pH 4.45, 4.50, 4.55 and 4.60 (Error bars present a pooled standard error of the mean, 0.252).

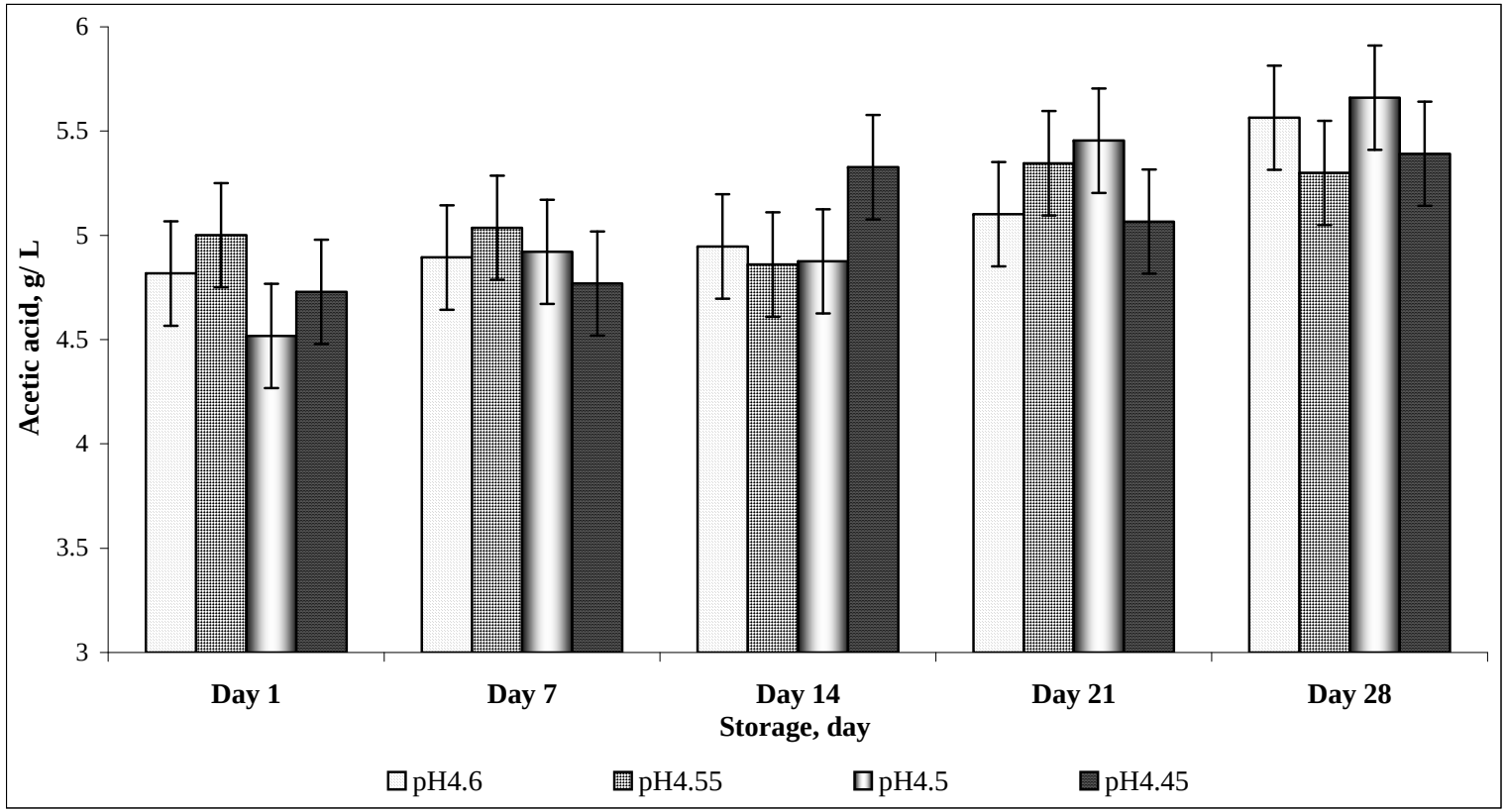

Figure 3.2b Production of acetic acid in probiotic yoghurt by yoghurt and probiotic cultures during refrigerated storage at different termination pH 4.45, 4.50, 4.55 and 4.60 (Error bars present a pooled standard error of the mean, 0.252).

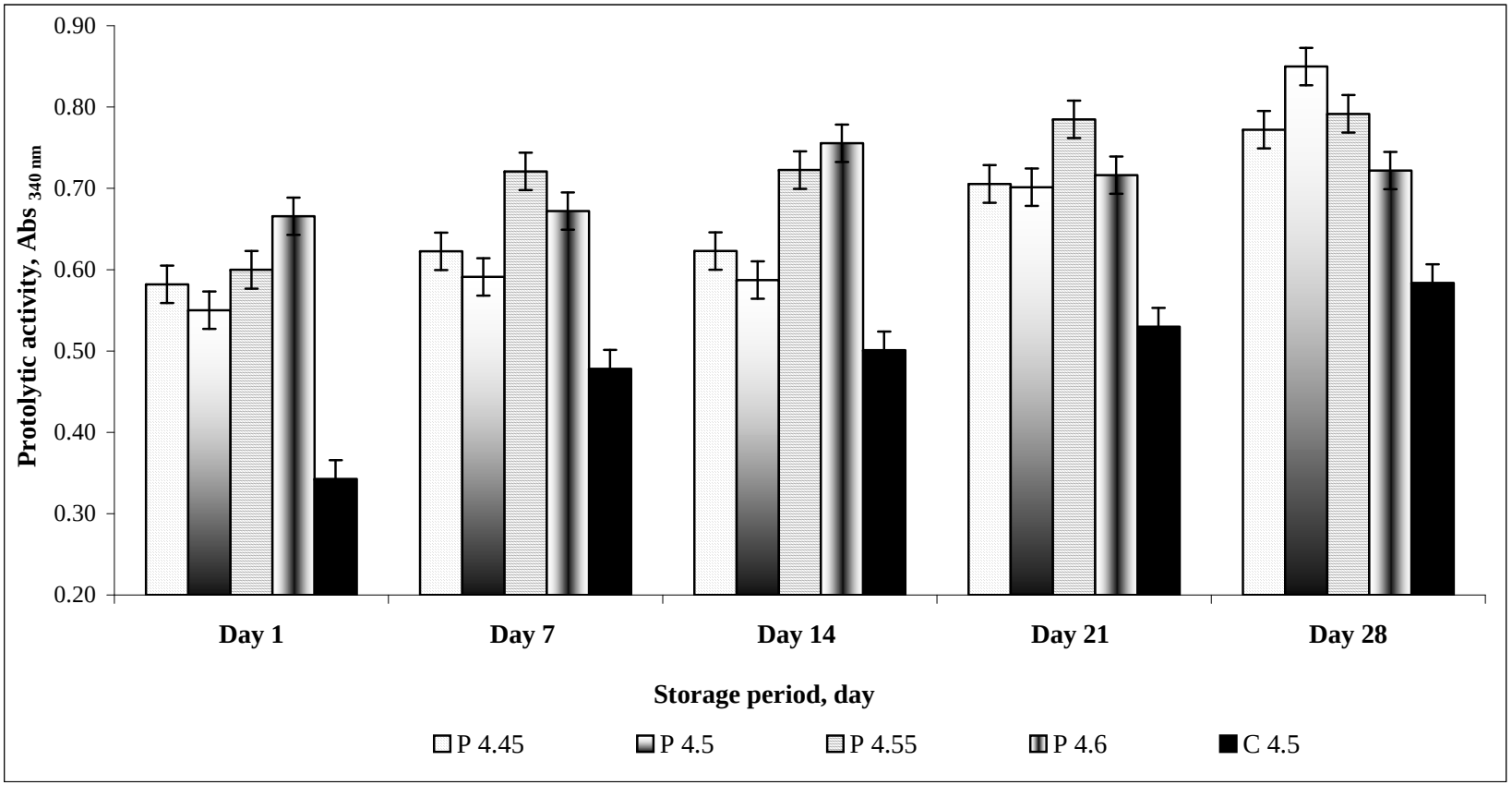

Figure 3.3 Proteolytic activity of yoghurt and probiotic cultures in yoghurt batches produced at different termination pH during cold storage; P = Probiotic; C = Control (Error bars present a pooled standard error of the mean, 0.023).

4.0 Probiotic strains as starter cultures improve angiotensin-converting enzyme inhibitory activity in soy yoghurt

4.1 INTRODUCTION

Soymilk based yoghurt offers a considerable appeal for a growing segment of consumers with certain dietary and health concerns. It has several nutritional advantages over cow's milk yoghurt including reduced level of cholesterol and saturated fat as well as no lactose (Favaro Trindade *et al*., 2001). Additionally, soy products contain the isoflavone phytoestrogens with potential anticarcinogenic activity (Xu *et al*., 1994; Wiseman 1997; Setchell and Cassidy 2003). On the other hand, soymilk, which serves as a base for a variety of beverages, contains indigestible oligosaccharides such as raffinose and stachyose, which are usually associated with stomach discomfort and flatulence (Rackis *et al*., 1970). The presence of pentanal and n-hexanal also imparts the characteristic undesirable bean flavor to soymilk. A wide variety of soy products offered on the market include soy-based cheeses, tofu, soy yoghurt, soy ice cream, soy sauce and soy vegetable burgers. Also soybean flour, soy protein concentrates and isolated soy proteins are some of ingredients used in the food industry (Wiseman 1997; Liu 1997). Many improvements have been implemented in the processing conditions and methods of preparation of soymilk to improve functionality and usability of soy proteins in a variety of products; for example, prolonged soaking of soybeans results in increased loss of carbohydrates thus minimizing the amount of fermentable sugars and subsequently avoiding gastrointestinal discomfort (Wiseman 1997; Liu 1997).

Fermentation of soymilk offers not only a means of preserving soymilk but also a possibility for modifying or improving its flavour and texture as well as enhancing its beneficial health properties (Liu 1997; Tsangalis *et al*., 2004). It also leads to new types of soy products resembling cultured dairy products (Liu 1997). Soymilk is a good medium for growing *Bifidobacterium* sp. due to presence of sucrose, raffinose and stachyose, which are fermented by most of the strains belonging to this genus (Kamaly 1997; Liu 1997; Scalabrini *et al*., 1998). The hydrolysis of these α-D-galactosyl oligosaccharides requires α-galactosidase activity, which hydrolyses α-galactoside bond found in these indigestible sugars (Rackis *et al*., 1970; Scalabrini *et al*., 1998). Other lactic acid bacteria (LAB) thrive well in soymilk, but produce less acid than in bovine milk, mainly because soymilk lacks free monosaccharides and disaccharide lactose (Liu 1997). As early as 1934, Kellogg patented a method to produce a butterlike product by fermenting soymilk with *L. acidophilus* (Liu, 1997). Later, many workers successfully prepared soy-based yoghurt by

Donkor, O. N., Henriksson, A., Vasiljevic, T., & Shah, N. P. (2005). Probiotic strains as starter cultures improve angiotensin-converting enzyme inhibitory activity in soy yoghurt. *Journal of Food Science*, *70*, M375-M381.

lactic acid fermentation (Angeles and Marth 1971). Recently, the use of LAB in preparing fermented soy products has received increased attention (Lee *et al*., 1990; Cheng *et al*., 1990; Karleskind *et al*., 1991). Although some of the products have not become available on the market, the idea and approach have shown promising results (Liu 1997). Importantly, the growth of LAB in soymilk is governed by the presence of fermentable sugars. Different workers have observed an increase in acid production after addition of simple sugars such as lactose, sucrose, glucose, and galactose, and/or enrichment with milk solids (Pinthong *et al*., 1980; Karleskind *et al*., 1991). In general, a 1% level of lactose fortification improves bacterial growth significantly (Liu 1997).

Lactobacillus acidophilus, *Bifidobacterium* sp. and *Lactobacillus casei* are classified as probiotics since they are thought to exert beneficial health effects in the host by modulating the intestinal microflora (Schrezenmeir and De Vrese 2001). A number of health benefits attributed to probiotics include antimicrobial, antimutagenic, anticarcinogenic and antihypertensive properties (Scheinbach 1998; Shah 2000a; Schrezenmeir and De Vrese 2001; Lourens-Hattingh and Viljoen 2001). Antihypertension has been reported to be mediated through inhibition of angiotensin converting enzyme (ACE; EC 3.4.15.1) (Nakamura *et al*., 1995). This enzyme plays a major role in the regulation of blood pressure. ACE converts angiotensin I into angiotensin II, a highly potent vasoconstrictor, with simultaneous inactivation of bradykinin, a vasodilatory peptide involved in local flow regulation (Saito *et al*., 2000; Fuglsang *et al*., 2003). Many ACE inhibitory peptides have been derived from not only milk proteins but also from other food proteins (Saito *et al*., 2000; Wu and Ding, 2001). Recently, a Finish dairy company introduced a fermented product, called Evolus, containing *L. helveticus* with a potent ACE inhibitory activity (Korpela, 2003). At present, detailed information is missing in the literature about the behavior of probiotic organisms (especially *L. acidophilus* and *L. casei*) in soymilk and the importance of these organisms as part of the starter cultures for making fermented soy products.

This study was undertaken to assess: (a) suitability of soymilk as a substrate for growth and acid development by selected probiotic strains at different termination pH; and (b) proteolytic and ACE-inhibitory activities of selected microorganisms.

4.2 MATERIALS AND METHODS

4.2.1 Bacterial strains and culture conditions

Propagation of cultures was performed according to the method described in Section 3.2.2. The resultant activated organisms were used for the production of cultures for making soy yoghurt. The cultures were prepared by inoculating each of 10 mL aliquots of sterilized Sanitarium Organics (simply soy, Sanitarium NSW, Australia) soymilk obtained from a local supermarket, supplemented with 1% lactose (Sigma) in order to improve the rate of acidification.

4.2.2 Soy yoghurt preparation and storage

One percent of α-lactose monohydrate (w/v) (Sigma) was added to three litres of soymilk to enhance bacterial growth and increase acid production (Liu 1997; Kamaly 1997). The mixture was heat treated in a water bath at 95^{o}C for 30 min. The soymilk was allowed to cool to 42^{o}C and was aseptically inoculated with 1% each of Lb1466 and St1342. The inoculated soymilk was divided into two portions; one portion was used as a control and the other portion was aseptically inoculated with 1% (v/v) of each probiotic cultures (L10, B94 and L26). The control and the probiotic portions were subsequently sub-divided into three batches respectively. All batches were held at 42^{o}C until the required pH of 4.50, 4.55 or 4.60 was reached, at which the fermentation was terminated. The pH change was monitored by a pH meter (model 8417, HANNA Instruments, Singapore). Soy yoghurts were immediately cooled after fermentation on ice bath and stored at 4^{o}C for 28 days. The pH of all batches of yoghurt was measured at termination of fermentation, then 12 h post-fermentation, and subsequently at 7 day intervals for 28 days.

4.2.3 Determination of viability

The colony counts of *L. delbrueckii* ssp. *bulgaricus* Lb1466, *S. thermophilus* St1342, *L. acidophilus* L10, *B. lactis* B94 and *L. casei* L26 in soy yoghurt during cold storage at day 1, 7, 14, 21 and 28 were determined according to the procedures described in Section 3.2.4.

4.2.4 Quantification of organic acids

Quantification of organic acids was performed according to the method described in Section 3.2.5.

4.2.5 Determination of proteolytic activity

Proteolytic activities of cultures used in the production of all batches of soy yoghurts were assessed according to the procedure described in Section 3.2.6.

4.2.6 Determination of ACE inhibitory activity

Approximately 25 g of soy yoghurt sample from each batch was centrifuged at 4000 x g for 15 min at 4^{o}C. The supernatant was collected and pH was subsequently adjusted to 8.3 using 10 M NaOH. The suspension was then centrifuged for 5 min at 14000 x g at 4^{o}C. The supernatant was collected again and freeze dried using a Dynavac freeze drier (Model FD300; Dynavac Eng. Pty. Ltd., Melbourne, Australia) for 24 h. Approximately 0.25 g of the lyophilized powder was dissolved in 3 mL of deionised water. The resulting solution was characterised for its inhibitory activity towards rabbit lung ACE using the method of Cushman and Cheung (1971) and Roy *et al*. (2000). An aliquot (200 μL) of hippuryl-histidyl-leucine (Sigma) buffer (5 mM Hip-His-Leu in 0.1 M borate buffer containing 0.3 M NaCl, pH 8.3) was mixed with 60 μL of sodium borate buffer and pre-incubated with 20 μL of the inhibitory solution for 5 min at 37^{o}C. The reaction was initiated by adding 20 μL of ACE solution (buffer containing ACE at a concentration of 0.1 unit/mL) followed by incubation at 37^{o}C for 30 min. The reaction was stopped by adding 250 μL of 1 M HCl and mixed with 1.7 mL of ethyl acetate. The mixture was centrifuged and 1.4 mL of the organic phase (ethyl acetate) was transferred to a fresh test tube. The ethyl acetate was evaporated to dryness on a water bath for 30 min at 100^{o}C. The residue containing hippuric acid was dissolved in 1 mL of deionised water and the absorbance of the solution was measured spectrophotometrically using a Cary IE UV/visible spectrophotometer (Varian Australia Pty. Ltd., Melbourne, Australia) at 228 nm against water as a blank. The extent of inhibition was calculated as follows:

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

where A is the absorbance in the presence of ACE and without the ACE-inhibitory component, B is the absorbance without ACE-inhibitory component, C is the absorbance with ACE and ACE-inhibitory component and D is the absorbance without ACE and with the ACE-inhibitory component. ACE inhibition was also expressed in terms of IC_{50}, defined as the sample concentration (mg/mL) required to inhibit 50% of the ACE activity in this study. All determinations were carried out in triplicate.

4.2.7 Statistical analysis

Statistical analyses of all data obtained were performed as described in Section 3.2.7.

4.3 RESULTS

4.3.1 Viability

Low levels of fermentable sugars and likely absence of some other growth factors extended the fermentation time required to obtain a desired termination pH. On an average, the fermentation time ranged between 6 h to 6 h 50 min for probiotic soy yoghurt and 9 h to 9 h 50 min for the control soy yoghurt, substantially longer than those observed in the bovine milk based yoghurt (Section 3.2.3). The concentration of all strains in soymilk upon inoculation ranged between 7.38 to 7.50 cfu/mL. The cell concentration in general increased significantly ($P < 0.05$) during fermentation, reaching values that were, on average, one log cycle higher at the end of the fermentation. In general, all cultures reached a cell concentration above 10^8 cfu/mL (Table 4.1). Apparently, probiotic cultures had a significant ($P < 0.05$) effect on the yoghurt culture in improving its concentration at the termination of fermentation and maintaining its high viability throughout the cold storage. As presented in Table 4.1, the cell concentration of St1342 in the probiotic soy yoghurts was significantly ($P < 0.05$) higher in comparison to that assessed in the control soy yoghurt batches. This organism maintained constant cell counts in all batches containing probiotics during storage as opposed to that in the control soy yoghurts, which experienced slight but not significant ($P > 0.05$) decline in cell counts from day 21 to 28. On the other hand, Lb1466 experienced a significant ($P < 0.05$) decline in the cell concentration in probiotic soy yoghurts after 3 weeks of storage.

All probiotic strains grew well in soy yoghurt reaching therapeutic levels above 10^8 cfu/mL. In general, L10 did not show any change in cell counts from day 1 to day 28. Similarly, there was no significant ($P > 0.05$) decrease in the cell counts of B94 during storage, although a slight but not significant ($P > 0.05$) decline in cell counts for probiotic batch terminated at pH 4.50 was recorded on day 28. Surprisingly, L26 showed variable cell viability during cold storage. The culture achieved the cell concentration of 8.0 log cfu/mL, which remained constant during first week of storage. By day 14, the cell concentration declined by one log cycle ($P < 0.05$), subsequently recovering ($P < 0.05$) and achieving concentrations over 8.0 log cfu/mL. Viability of probiotics in soy yoghurts, in general, did not vary significantly ($P > 0.05$). Similarly the termination pH had no effect on viability of probiotic organisms among batches, although overall, L10 showed the highest growth (Table 4.1).

4.3.2 pH changes in the batches of soy yoghurts

The pH of all batches of soy yoghurt appeared to be strain dependant. As shown in Table 4.2, the pH was maintained constant in the control soy yoghurt during first two weeks of cold storage. On the other hand, the change in pH of probiotic soy yoghurt occurred after first week. By the end of storage period, the pH of all products was significantly ($P < 0.05$) lower than that recorded at the termination of fermentation and ranged between 0.08 and 0.13 and between 0.19 to 0.21 in control and probiotic soy yoghurt, respectively. Furthermore, the termination pH had no effect ($P > 0.05$) on the extent of pH decline during cold storage.

4.3.3 Organic acid content

Organic acids produced during cold storage of soy yoghurt were mainly formed due to supplementation with 1% lactose. The results obtained by HPLC analysis are presented in Figures 4.1 and 4.2. Lactic acid content in the control soy yoghurt increased steadily during storage. The increasing trend in lactic acid production of the probiotic soy yoghurts was similar to that of the control batches reaching similar levels (0.24 g/L) by the end of storage (Figure 4.1). Pinthong *et al*. (1980) and Favaro Trindade *et al*. (2001) also observed an increase in acid production after addition of glucose and sucrose, respectively. Probiotic soy yoghurt at the termination pH of 4.50 appeared to contain the highest amount of lactic acid among all batches examined, as the concentration was significantly ($P < 0.05$) different from

that found in other batches during storage. However, lactic acid concentration leveled off in all three batches ($P > 0.05$) by the end of storage.

Acetic acid was determined in all batches with control batches containing significantly ($p < 0.001$) lower concentration than those samples with probiotics (Figure 4.2). All control batches obtained at different termination pH contained a similar concentration of acetic acid without a statistical difference ($P > 0.05$). Thus, results for the control soy yoghurt obtained by termination of fermentation at pH 4.50 are only presented. The concentration of acetic acid in the control soy yoghurt increased significantly ($P < 0.05$) during first two weeks of storage and remained constant until day 28. Different termination pH initially appeared to have a substantial effect on the degree of acetic acid production in the batches containing probiotics (Figure 4.2). Probiotic soy yoghurt produced by terminating fermentation at pH 4.50 had the highest concentration of acetic acid and the concentration remained constant during storage. Although, concentration of acetic acid in the batches of probiotic soy yoghurt at pH 4.55 was significantly ($P < 0.05$) lower in comparison to that of those at pH 4.50, it increased substantially during the last two weeks of storage, subsequently resulting in no observable difference ($P > 0.05$) in the acetic acid content recorded for these two batches. Surprisingly, the batch obtained at pH 4.60 contained lowest acetic acid content, not significantly ($P > 0.05$) different from that recorded in the control batch.

4.3.4 Proteolysis in soy yoghurt

Proteolytic activity of selected probiotic strains as well as yoghurt culture in soy yoghurt during prolonged cold storage was estimated by determination of free amino groups and presented in Figure 4.3. The OPA-based spectrophotometric detection of released amino groups resulting from the proteolysis of soymilk proteins gave a direct measurement of proteolytic activity. The extent of proteolysis based upon OPA values was time dependant and varied among batches ($P < 0.05$). The termination pH appeared to play a role in the proteolytic activity of studied organisms but its effect varied greatly and did not follow any trend. As expected, the extent of proteolysis increased during storage for all batches including the control soy yoghurt. Noticeably, the soy yoghurt products containing probiotics and obtained by termination at pH 4.55 and 4.60 contained significantly ($P < 0.05$) higher levels of liberated amino groups in comparison to other batches. These two batches recorded the absorbance values of 0.033 and 0.046, respectively, after two weeks of storage. Consequently, these values increased reaching absorbance values of 0.067 and

0.054, respectively, at the end of storage. These findings were consistent with those reported by Nielsen *et al*. (2001) and Leclerc *et al*. (2002) on the amount of free NH_3 groups increasing with fermentation time.

4.3.5 ACE-inhibitor activity

All soy yoghurt products with or without probiotics expressed in vitro ACE inhibitory activity illustratively presented in Figure 4.4. The ACE inhibitory activity of all batches of probiotic soy yoghurt showed very similar trends with no significant difference ($P > 0.05$). Thus, only results for control and probiotic containing soy yoghurt obtained at pH 4.5 are presented. Production of ACE inhibitors was likely not confined to a single species of bacterium. In general, all batches exerted ACE inhibitory activity but in varying degrees. The degree of the ACE inhibition by soy yoghurt produced with starter culture containing probiotic organisms was significantly ($P < 0.05$) greater than that of the control culture consisting solely of the yoghurt culture (Figure 4.4). Furthermore, the arbitrary measurement of relative peptide content by OPA method showed that the amount of free amino groups formed in the soymilk during fermentation was strain-dependant as reported previously (Fuglsang *et al*., 2003). Statistical analysis revealed a slight positive correlation ($r = 0.60$) between the OPA results and ACE inhibition, indicating that the extent of ACE inhibition might have been partially dependant on the extent of the proteolytic activity.

4.4 DISCUSSIONS

To effectively modulate intestinal microbial balance and potentially enhance the health benefits, it is essential that probiotics be alive in sufficient numbers in soy yoghurt at the time of consumption. It has been suggested that probiotic bacteria be present in cultured milk or soymilk to a minimum level of 6 log cfu/mL in order to provide a therapeutic dose (Gomes and Malcata 1999). Strains used in our study successfully attained a desired level, achieving at least 8.0 log cfu/mL of each strain in soy yoghurt. Viability of probiotic organism in soy yoghurt batches was not affected by the different termination pH of fermentation at 4.60, 4.55 and 4.45. The trend was similar for the control batch even though there were slight decreases in cell counts in both the control and probiotic yoghurts during storage. The decreases in cell concentration for all batches including control at the end of storage was probably due to a significant ($p < 0.0001$) decline in pH caused by increased concentration of organic acids (lactic and acetic acids) (Figure 4.1). More importantly, even though concentration of organic acids increased during storage, probiotic strains, L10, B94,

L26 and starter cultures, Lb 1466 and St 1342, in probiotic soy yoghurts maintained appreciable viability. Omafuvbe *et al*. (2002) reported a similar pattern in the fermentation of soybean by *Bacillus* species. There was low production of lactic (0.17-0.24 g/L) and acetic (0.01-0.02 g/L) acids in soy yoghurt during storage compared to yoghurt, in which lactic acid content ranged between 13.91 and 15.92 g/L during storage. This confirmed previous observation (Section 3.4) that lactic acid would affect viability of some strains of probiotic bacteria. LAB appear to grow well in soymilk, but produce less organic acid than in bovine milk. Liu (1997) also reported similar observations along with some other authors suggesting soymilk as an appropriate growth medium for some LAB (Angeles and Marth 1971; Kamaly 1997). The decline in the cell concentration during prolonged cold storage, such as in case of Lb 1466, could be attributed to the competition for nutrients. On the other hand, probiotic soy yoghurt appeared to have supported the growth of Lb 1466 and St 1342 during storage in comparison to the control batches. Thus, nutrients may not have been the only factor determining viability but other growth factors such as free NH_3 groups and peptides in the probiotic soy yoghurt may have played a role in maintaining the viability of all cultures.

The increase in acidification in the probiotic soy yoghurt was mainly due to the growth of Lb1466 and St1342 during storage as recorded in the control soy yoghurt (Figure 4.1) (Favaro Trindade *et al*., 2001). Even though acetic acid was produced during storage in both the probiotic and the control batches, those containing probiotic cultures had slightly more acetic acid (about 1-2% more) than the control batches produced likely by probiotics.

The study showed that probiotic organisms as well as Lb1466 and St1342 were proteolytic in soy yoghurt during growth and that the degree of proteolysis depended significantly ($p<0.0005$) on storage time. Kamaly (1997) showed that *Bifidobacterium* strains exhibited more pronounced proteolytic activity in soymilk than that in RSM. The primary enzymes in LAB responsible for the hydrolysis of the proteins are proteinases and peptidases (Law and Haandrickman, 1997; Shihata and Shah 2000). As growth of probiotic organisms was maintained in soy yoghurts, bacterial activities including proteolysis increased (Figure 4.3). The increased proteolytic activity may have improved the level of growth factors in the form of free NH_3 groups and peptides. This finding was in line with the results of Kamaly (1997) and Nielsen *et al*. (2001) on proteolysis in soymilk. Shihata and Shah (2000) recorded a high proteolytic activity of several strains of *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus* in yoghurt, which was apparently strain dependent. This activity was also substantially higher from that observed for some of selected probiotic

strains including *Bifidobacterium* and *L. acidophilus*. In our current study, Lb1466 and St1342 showed a very low proteolytic activity during growth and cold storage in soy yoghurt in comparison to that reported by Shihata and Shah (2000) likely due to absence of hydrolysable substrate in the form of milk proteins (Kunji *et al*., 1996).

Peptides released during fermentation are not always completely used by bacteria for their growth (Leclerc *et al*., 2002) and sometimes large amounts can be accumulated in the medium. These peptides are measured using the OPA method (Nielsen *et al*., 2000). Among LAB, *L. helveticus* has been shown to possess strong proteolytic activity in milk-based media and was also known to produce potent ACE-inhibitory peptides during milk fermentation (Yamamoto *et al*., 1994a; Leclerc *et al*., 2002). Due to the strong proteolytic activity of selected LAB strains in soy yoghurt observed in the current study, the work was undertaken to assess the potency of the hydrolyzed proteins to act as ACE inhibitors in vitro (Figure 4.4). The magnitude of inhibition was not as high as recorded with clinically used inhibitors such as captopril (Mullally *et al*., 1997; Vermeirssen *et al*., 2002). The low levels of inhibition may indicate that fermented milk and soymilk do not possess the same range of hypotensive potentials as the classical inhibitors. However, Hata *et al*. (1996) have shown that LAB may have a clinical potential in preventing cardiovascular disease in mildly hypertensive humans. Our findings showed that mixed LAB culture used in the current study produced ACE inhibitors during soymilk fermentation resulting in an IC_{50} value of 0.28 IC_{50} mg/mL and 69.7% ACE inhibition at the end of storage period (Figure 4.4). Similarly, Pedroche *et al*. (2002) reported ACE-inhibitory activity of chickpea protein hydrolysates having an IC_{50} value of 0.19 mg/mL. Suh *et al*. (1999) reported the potential of corn gluten hydrolysate to suppress the ACE activity resulting in 84.3% inhibition. Several studies have shown a potency of soy protein hydrolysates, obtained mainly by alkaline hydrolysis, to lower high blood pressure in hypertensive rats (Wu and Ding 2002; Yang *et al*., 2004). Kuba *et al*. (2003) reported an IC_{50} value of 1.77 mg/mL recorded for peptides found in tofuyo fermented soybean food. Based on the findings in the current study, it would likely be expected to observe more pronounced ACE inhibitory effect by consumption of soy yoghurt than that of tofuyo.

4.5 CONCLUSIONS

This study showed that lactose supplemented soy yoghurt may be a suitable medium for a delivery of probiotics. In general, all selected probiotic cultures, L10, B94 and L26, showed appreciable viability, above therapeutic minimum, during prolonged cold storage,

which is an additional possible beneficial effect of soy yoghurt. Different termination pH appeared to have no effect on the viability of probiotic organisms in soy yoghurt and survival of probiotic organisms was not strain dependant since all strains showed good cell counts. The use of yoghurt culture in conjunction with probiotic cultures resulted in the appreciable proteolytic activity likely improving the growth of selected probiotics. More importantly, soy yoghurt produced by probiotic strains as adjunct culture exerted appreciable ACE inhibitory activity. The enhanced proteolytic activity might have contributed to the observed inhibition, although contributions from other metabolites should not be disregarded. The development of soy yoghurt containing higher concentration of released bioactive ACE inhibitors and viable probiotics may deliver health benefits of these functional compounds more efficiently.

Table 4.1 Viability of selected probiotic organisms and yoghurt culture in soy yoghurt batches supplemented with 1% lactose and produced by controlled fermentation and terminated at different pH of 4.50, 4.55 and 4.60 during 28 day of storage at 4°C

Culture	Termination pH	Cell concentration, log_{10} cfu/g					
		Period of storage, day					
		0	1	7	14	21	28
Control batches							
Lb1466	4.60	7.38^{Aa}	8.21^{Ab}	8.50^{Ab}	8.46^{Ab}	8.20^{Abc}	7.96^{Abc}
	4.55	7.38^{Aa}	8.39^{Ab}	8.49^{Ab}	8.45^{Ab}	8.23^{Abc}	8.07^{Abc}
	4.50	7.38^{Aa}	8.41^{BAb}	8.56^{Ab}	8.41^{Abc}	8.23^{Abcd}	8.07^{Abcd}
St 1342	4.60	7.48^{Aa}	$7.96^{A\,b}$	$7.93^{A\,b}$	$7.91^{A\,b}$	7.91^{Ab}	7.90^{Ab}
	4.55	7.48^{Aa}	8.22^{Bb}	8.09^{Bb}	7.92^{Ab}	8.15^{Ab}	8.00^{Ab}
	4.50	7.48^{Aa}	8.19^{CBb}	8.11^{CBb}	7.91^{Ab}	8.18^{Ab}	8.19^{Bb}
Probiotics batches							
Lb1466	4.60	7.44^{Aa}	8.48^{Aab}	8.85^{Abc}	8.57^{Ab}	7.97^{Abcd}	7.63^{Aaac}
	4.55	7.44^{Aa}	8.52^{Ab}	8.93^{Abc}	8.85^{Abc}	7.97^{Abcd}	7.57^{Aac}
	4.50	7.44^{Aa}	8.66^{BCb}	8.88^{Ab}	8.90^{Ab}	8.39^{Aab}	8.12^{Aab}
St 1342	4.60	7.51^{Aa}	8.35^{Ab}	8.50^{Ab}	8.55^{Abc}	8.59^{Abc}	8.57^{Abc}
	4.55	7.51^{Aa}	8.30^{Ab}	8.45^{Ab}	8.45^{Abc}	8.59^{Abc}	8.53^{Abc}
	4.50	7.51^{Aa}	8.23^{Ab}	8.53^{Ab}	8.46^{Ab}	8.45^{BCb}	8.30^{Ab}
LAFTI®L10	4.60	7.46^{Aa}	8.81^{Ab}	8.95^{Ab}	8.89^{Ab}	8.76^{Abc}	8.58^{Abc}
	4.55	7.46^{Aa}	8.76^{Aa}	8.89^{Aa}	8.84^{Aa}	8.81^{Aa}	8.51^{Aa}
	4.50	7.46^{Aa}	8.81^{Ab}	8.93^{Ab}	8.18^{Ab}	8.79^{Abc}	8.57^{Abc}
LAFTI®B94	4.60	7.51^{Aa}	8.67^{Ab}	8.35^{Abc}	8.48^{Ab}	8.53^{Ab}	8.44^{Ab}
	4.55	7.51^{Aa}	8.58^{Ab}	8.25^{Abc}	8.49^{Ab}	8.54^{Ab}	8.25^{Abc}
	4.50	7.51^{Aa}	8.52^{Aa}	8.28^{Aa}	8.43^{Aa}	8.25^{Aa}	7.86^{Aa}
LAFTI®L26	4.60	7.50^{Aa}	8.56^{Ab}	8.52^{Ab}	7.81^{Aab}	8.62^{Ab}	8.50^{Ab}
	4.55	7.50^{Aa}	8.57^{Ab}	8.42^{Ab}	7.75^{Aab}	8.54^{Ab}	8.36^{Ab}
	4.50	7.50^{Aa}	8.54^{Ab}	8.47^{Ab}	7.83^{Aa}	8.46^{Ab}	8.33^{Ab}
SEM				0.19			

Results presented as a mean of three observations. SEM - pooled standard error of the mean (0.19) Preset $p< 0.05$ Lb1466 = *L. delbruekii* ssp. *bulgaricus*, St1342 = *S. thermophilus*, LAFTI® L10 = *L. acidophilus*, LAFTI® B94 = *B. lactis* LAFTI® L26 = *L. paracasei*. ^{abcd} Means in the same row with different small letter superscripts are significantly different; ^{ABC} Means in the same column for a particular strain with different capital letter superscripts are significantly different.

Table 4.2 The level of pH decrease during prolonged cold storage of control and probiotic-containing soy yoghurt batches obtained by supplementation of 1% lactose and controlled fermentation terminated at different pH of 4.50, 4.55 and 4.60

Soy yoghurt	Time of storage, day	pH change, ΔpH		
		Termination pH		
		4.60	4.55	4.50
Control (yoghurt culture)	1	0.00 ^{Aa}	0.00 ^{Aa}	0.00 ^{Aa}
	7	0.00 ^{Aa}	0.00 ^{Aa}	0.00 ^{Aa}
	14	0.00 ^{Aa}	0.00 ^{Aa}	0.00 ^{Aa}
	21	0.05 ^{ACa}	0.04 ^{ACa}	0.12 ^{Ab}
	28	0.08 ^{BCa}	0.09 ^{BCa}	0.13 ^{Aa}
Probiotic (yoghurt + probiotic cultures)	1	0.00 ^{Aa}	0.00 ^{Aa}	0.00 ^{Aa}
	7	0.05 ^{Aa}	0.12 ^{Aa}	0.09 ^{Aa}
	14	0.08 ^{Aa}	0.14 ^{Aa}	0.08 ^{Aa}
	21	0.15 ^{Aa}	0.15 ^{Aa}	0.16 ^{BAa}
	28	0.20 ^{BAa}	0.21 ^{BAa}	0.19 ^{BAa}
SEM			0.03	

Yoghurt culture: *L. delbruekii* ssp. *bulgaricus* Lb1466, *S. thermophilus* St1342; Probiotic cultures: *L. acidophilus* LAFTI® L10, *B. lactis* LAFTI® B94 and *L. paracasei* LAFTI® L26. Results presented as a mean of three observations; SEM - pooled standard error of the mean (0.03); the level of significance was preset at p = 0.05. Means in the same row with different small letter superscripts are significantly different; Means in the same column for a particular batch with different capital letter superscripts are significantly different.

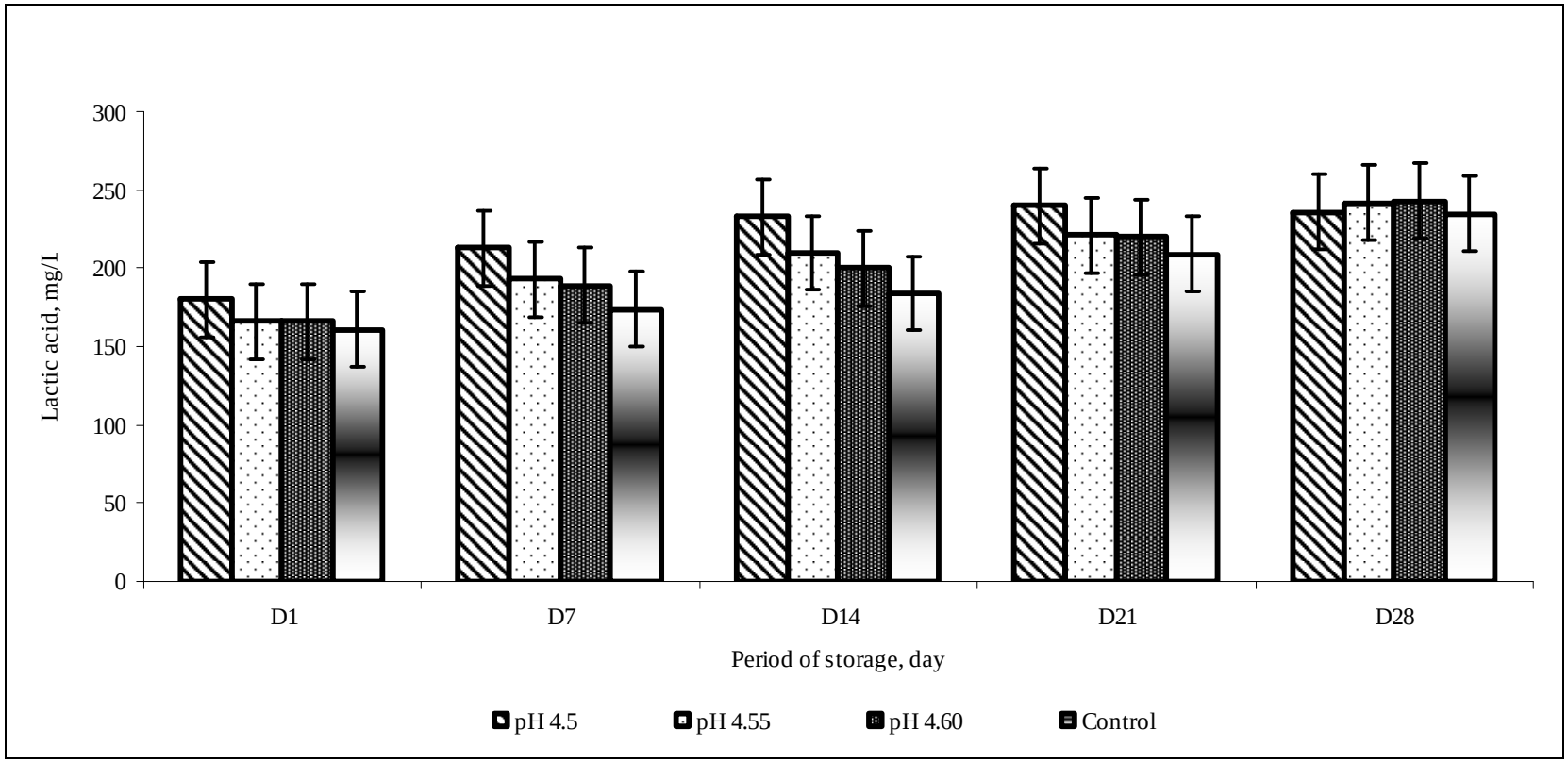

Figure 4.1 The change in lactic acid content generated by yoghurt starter culture with or without added probiotics in soy yoghurt, manufactured by controlled fermentation and terminated at different pH of 4.50, 4.55 or 4.60, during prolonged refrigerated storage (Error bars present a pooled standard error of the mean, SEM = 0.023 mg/mL).

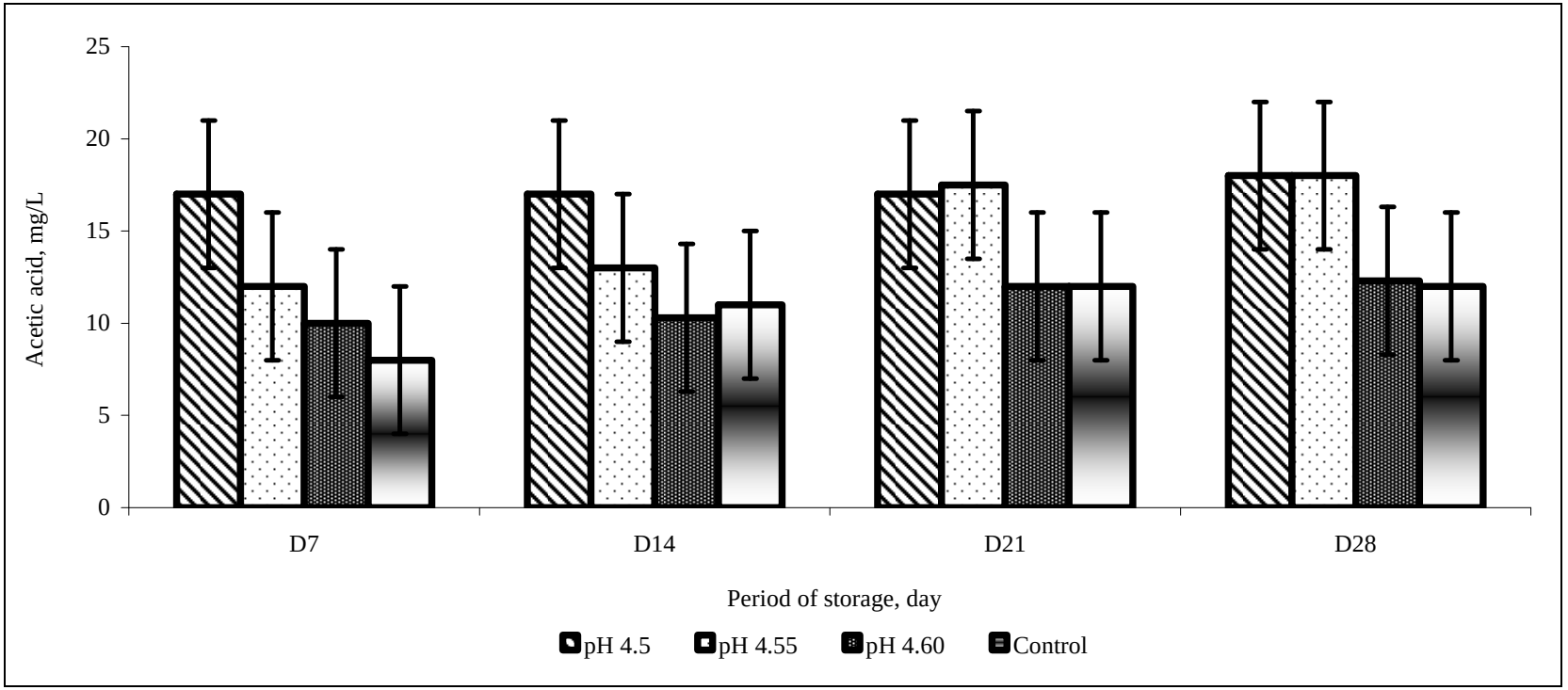

Figure 4.2 The change in acetic acid content generated by yoghurt starter culture with or without added probiotics in soy yoghurt, manufactured by controlled fermentation and terminated at different pH of 4.50, 4.55 or 4.60, during prolonged refrigerated storage (Error bars present a pooled standard error of the mean, SEM = 0.004 mg/mL).

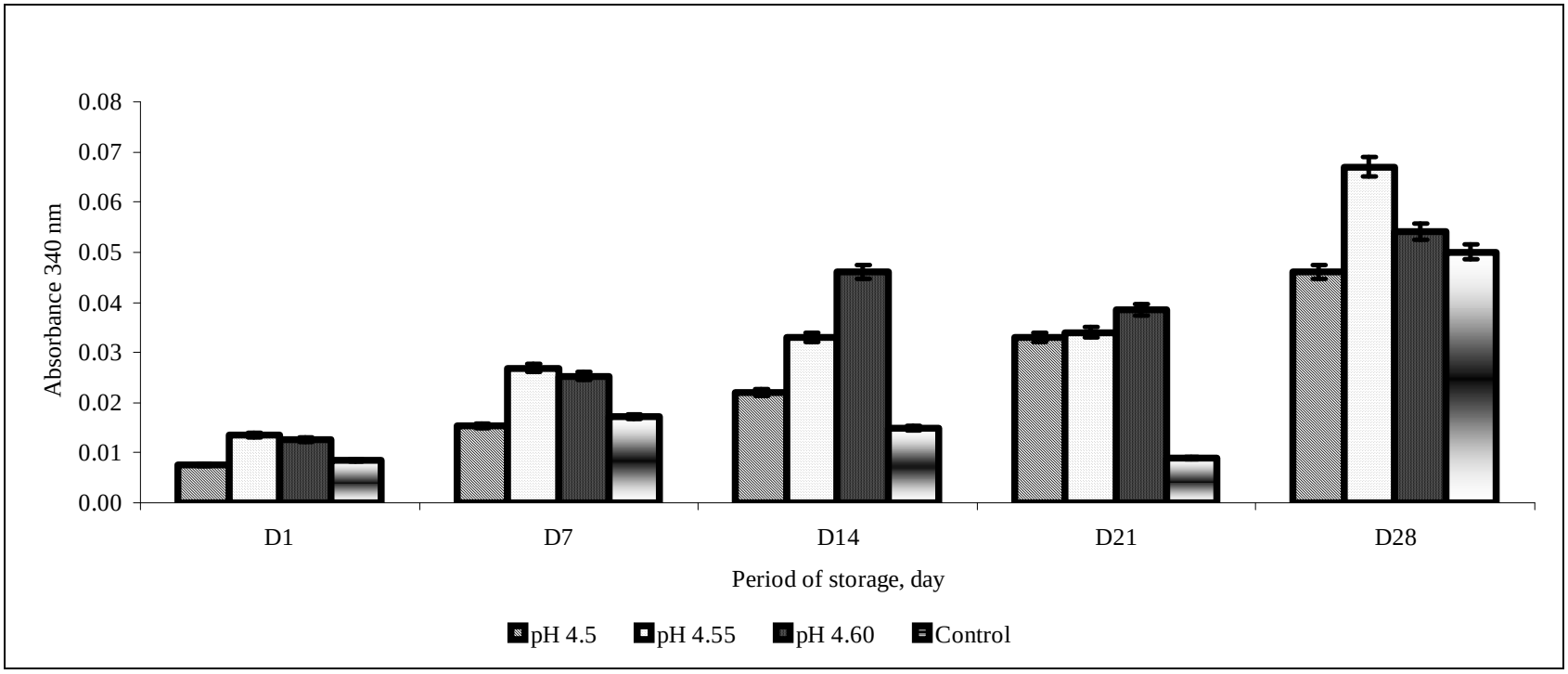

Figure 4.3 Proteolytic activity of yoghurt and probiotic cultures in control and probiotic soy yoghurts, supplemented with 1% lactose and produced by controlled fermentation terminated at different pH of 4.50, 4.55 and 4.60, during prolonged cold storage (Error bars present a pooled standard error of the mean, SEM = 0.012 mg/mL).

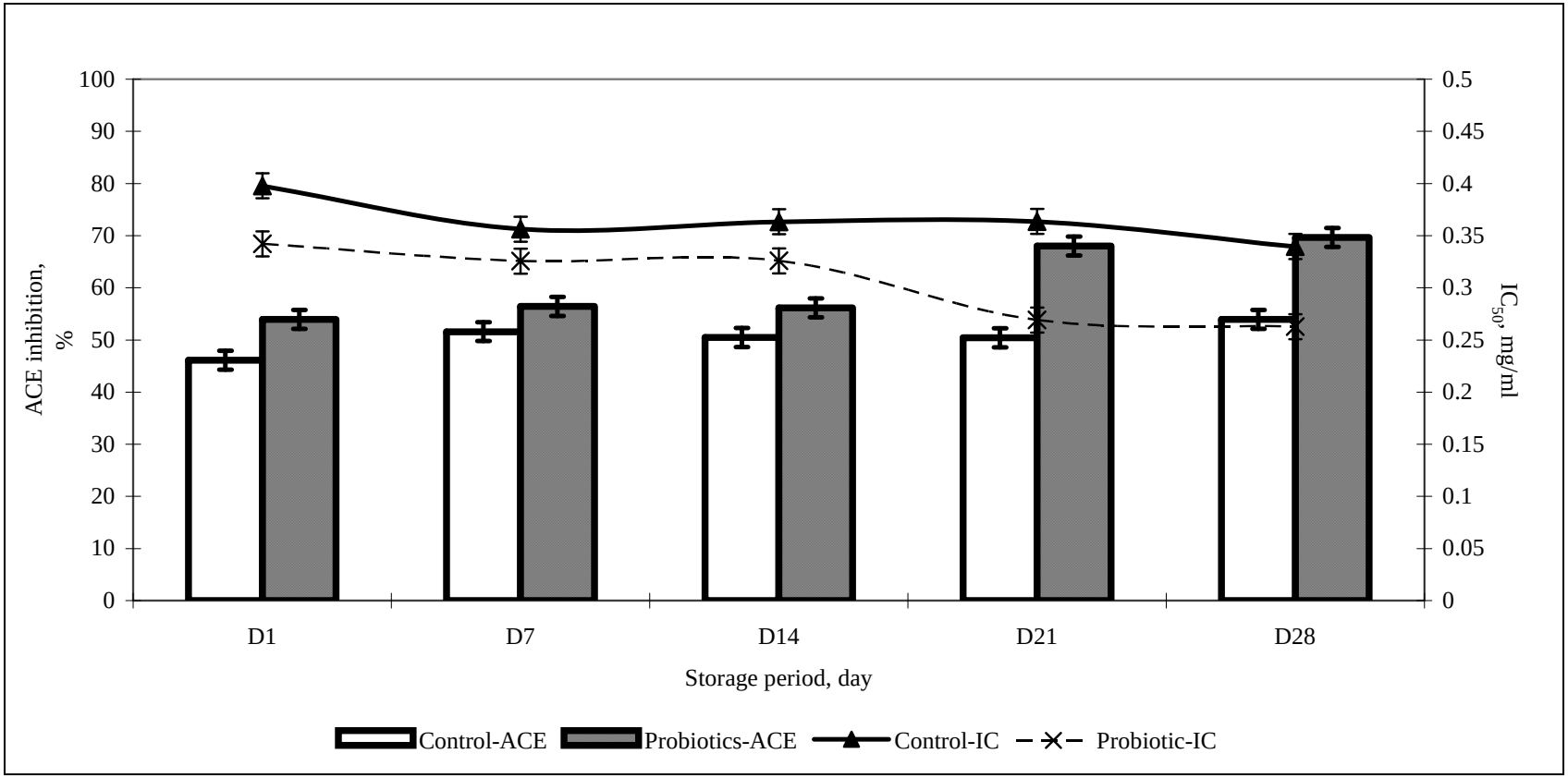

Figure 4.4 The extent of ACE-inhibitory activity and IC_{50}, mg/mL of yoghurt and probiotic cultures in soy yoghurt, supplemented with 1% lactose and produced by controlled fermentation terminated at pH 4.50, during 28 days of storage at 4°C (Error bars present a pooled standard error of the mean, SEM=1.81% for ACE inhibition and SEM=0.01 for IC_{50}, mg/mL).

5.0 Survival and activity of selected probiotic organisms in set-type yoghurt during cold storage

5.1 INTRODUCTION

The presence of live microorganisms, in particular lactic acid bacteria (LAB), in food has been traditionally associated with certain health benefits. Previous investigators have attributed such health effects to shifts in the intestinal microbial balance (Schrezenmeir and De Vrese, 2001). This shift is attributed to probiotics, live microorganisms which, when added to foods, beneficially restore microbial balance in the gut flora of the host (Gibson and Roberfroid, 1995). In order to exert any beneficial health impact, the concentration of probiotics in a product that serves as a delivery system needs to be high. However, no general agreement has been reached on the recommended levels and the suggested concentrations of probiotics frequently referred to as therapeutic levels ranged from 10^6 cfu/mL (Kurmann and Rasic, 1991) to over 10^7 or 10^8 cfu/mL (Lourens-Hattingh and Viljoen, 2001). The main obstacle for achieving and maintaining required levels is the strain-dependence of probiotic organisms and poor survival due to increased acidity and oxygen tension (Dave and Shah 1997; Shah, 2000b; Talwalkar and Kailasapathy, 2004) and nutrient depletion (Dave and Shah 1997; Shah, 2000b). In order to overcome these problems, different approaches have been used including culture selection (Lourens-Hattingh and Viljoen, 2001; Tuomola *et al*., 2001), microencapsulation (Capela *et al*., 2006), and addition of prebiotics (Bruno *et al*., 2002; Corcoran *et al*., 2004; Capela *et al*., 2006).

Prebiotics are non-digestible complex carbohydrates that selectively stimulate the growth and/or activity of bacteria in the colon and also beneficially affect the host (Gibson and Roberfroid, 1995). They may exert a protective effect towards selected probiotic bacteria in improving their survival and activity during storage of the product containing probiotics as well as passage through the upper parts of the gastrointestinal tract (GIT) (Kaplan and Hutkins, 2000; Roberfroid, 2001). A range of oligosaccharides has been tested in this regard (Kaplan and Hutkins, 2000; Roberfroid, 2001) with inulin and other fructo-oligosaccharides frequently employed in studies as they resist digestion by gastric acid and pancreatic enzymes *in vivo* (Cummings *et al*., 2001). In most instances, *Bifidobacterium* sp. have been the main focus of research (Shin *et al*., 2000; Bruno *et al*., 2002; Akalin *et al*., 2004), although lactobacilli have been used widely in dairy products and deserve attention due to their health-promoting effects (Shah, 2000b; 2001).

Donkor, O. N., Nilmini, S. L. I., Stolic, P. Vasiljevic, T., & Shah, N. P. (2007). Survival and activity of selected probiotic organisms in set-type yoghurt during cold storage. *International Dairy Journal*, *17*, 657-665.

Inulin is a non-digestible fructan frequently used as a functional food ingredient that offers a unique combination of interesting nutritional properties and important technological benefits (Suzuki and Chatterton, 1993). The unique aspect of inulin is in its β(2 →1) bonds connecting fructose monomers and preventing enzyme digestion, which in turns results in reduced caloric value and dietary fiber effect (Niness, 1999).

In contrast to inulin, amylose maize starch (Hi-maize®) is composed solely of glucose monomers and exhibits a range of qualities that strongly indicate its capability to act as a prebiotic in foods (Haynes and Playne, 2002; Brown *et al*., 1998). Fifteen years ago, digestion and absorption of starch in the small intestine as a normal phenomenon has been recognized (Cummings and Englyst, 1991). This starch fraction, commonly referred to as resistant starch, has been suggested for use as a prebiotic in probiotic compositions to promote the growth of some beneficial microorganisms such as *Bifidobacterium* (Brown *et al*., 1996). Apart from serving as a growth promoter, various species of bacteria produce a range of potentially beneficial short-chain fatty acids (SCFA) when allowed to ferment Hi-maize *in vitro* (Brown *et al*., 1998; Le Leu *et al*., 2005). Stabilizers, also known as hydrocolloids or gums, are frequently added to improve water-binding capacity and prevent syneresis (Syrbe *et al*., 1998). However, the interaction of hydrocolloids with milk proteins may result in either improvement or deterioration of textural properties.

Several investigations have focused on improving growth and activities of *Bifidobacterium sp.* with inulin and hi-maize starch (Brown *et al*., 1996; Shin *et al*., 2000; Bruno *et al*., 2002; Akalin *et al*., 2004); however, reports are lacking regarding effects of these prebiotics on *Lactobacillus* sp. While the addition of prebiotics may have functional benefits for probiotics and subsequently to consumers, this approach may have an effect on the textural characteristics of yoghurt, which may affect their sensory perception (LaTorre *et al*., 2003).

Therefore, the objectives of this study were: (i) to investigate the effect of addition of selected prebiotics (hi-maize corn starch powder or inulin) on survival of two selected probiotic organisms, *Lactobacillus casei*, and *L. acidophilus*, in yoghurt made using *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* during cold storage; (ii) to assess the metabolic activity of the organisms in the presence of the prebiotics during storage at 4°C; and (iii) to evaluate the textural characteristics of the yoghurts as affected by different levels of prebiotics addition.

5.2 MATERIALS AND METHODS

5.2.1 Propagation of cultures

Pure strains of *S. thermophilus* St1342 and *L. delbrueckii* ssp. *bulgaricus* Lb1466 were obtained from the Culture Collection of Victoria University (Werribee, Australia). *L. acidophilus* LAFTI® L10 and *L. paracasei* LAFTI® L26 were kindly provided by DSM Food Specialties (Moorebank, NSW, Australia). The full factorial design and coding of the experimentation is provided in Table 5.1. Each organism was stored at -80°C. Sterile 10 mL aliquots of de Man Rogosa and Sharpe (MRS) broth (Sigma Chemical Co., St Louis, USA) were inoculated with 1% (v/v) of each strain and incubated at 42°C for Lb1466 and 37°C for St1342, *L. acidophilus* L10 and *L. paracasei* L26. The activated organisms were used after three successive transfers for the preparation of mother cultures for yoghurt making. The cultures were prepared by inoculating 10 mL aliquots of reconstituted skim milk (RSM) supplemented with 2% (w/v) glucose and 1% (w/v) yeast extract.

5.2.2 Preparation of yoghurt supplemented with prebiotics

Commercial pasteurized and homogenized whole milk was fortified with 2% (w/w) total solids low heat SMP 34% protein (Murray Goulbourn Co-operative Co. Ltd., Brunswick, Australia). The yoghurt base was supplemented with (0.5, 1.0 or 1.5)% (w/v) of commercially available inulin (Raftiline® ST, ORAFTI, Oreye, SA, Australia) or hi-maize (high-amylose corn starch; Starch Australasia Ltd., Lane Cove, NSW, Australia). Each mix was then heat treated at 90°C for 30 min and cooled to approximately 40°C followed by inoculation with 1% (v/v) each of *S. thermophilus* St1342 and *L. delbrueckii* ssp. *bulgaricus* Lb1466 with or without the addition of 1% (v/v) *L. acidophilus* L10 or *L. paracasei* L26. Inoculated yoghurt bases were then poured into 70 mL sterile plastic cups and incubated at 42°C until the pH reached 4.50, followed by cooling to 4°C and storing for 4 weeks. Survival of microorganisms was expressed as log cfu/mL, metabolic activity measured as production of organic acids, and proteolytic activity were determined and apparent viscosity was measured 12 h post-fermentation and subsequently at weekly intervals for 4 weeks.

5.2.3 Determination of viability of probiotic organisms

The enumeration of *S. thermophilus* St1342 and *L. delbrueckii* ssp. *bulgaricus* Lb1466, *L. acidophilus* L10 and *L. paracasei* L26 was performed according to the procedures described in Section 3.2.4

5.2.4 Determination of organic acids

Determination of lactic, acetic, butyric and propionic acids was carried out using the method described initially by Shin *et al*. (2000) with some modifications as reported in Section 3.2.5.

5.2.5 Determination of proteolytic activity

Proteolytic activities of organisms used in the production of all batches of yoghurts were assessed according to the procedure described in Section 3.2.6.

5.2.6 Measurement of viscosity

The viscosity of the yoghurt batches was measured on day 1 and at weekly intervals during storage at 4^{o}C and all samples were treated at a constant shear rate (Fernandez-Garcia *et al*., 1998). Samples were tested using a T-spindle (T-E size) coupled to an LVTD digital viscometer (Brookfield Engineering Laboratories, Stoughton, MA, USA). The viscometer was set at constant revolutions of 12 rpm. The yoghurt was gently stirred for 20 s (20 continuous sweeps) before reading was recorded. All determinations were repeated at least twice on the same sample.

5.2.7 Statistical analysis

The experiments were organized as a random, full-factorial split-plot in time design exploring the influence of prebiotics and time as the main effects. All experiments were replicated and sub-sampled at least once (n = 4 or more). Data were analysed as described in Section 3.2.7. The level of significance was preset at $P \leq 0.05$.

5.3 RESULTS AND DISCUSSIONS

5.3.1 Viability of lactic acid bacteria during refrigerated storage

Prebiotics are usually added to dairy products to selectively stimulate the growth of selected probiotics such as *Bifidobacterium* sp. in the human intestine. Due to increased interest in lactobacilli as probiotics, the effect of prebiotic addition on the survival and metabolic activity of *L. acidophilus* L10 and *L. paracasei* L26 was investigated during 28 day storage period at 4°C. Figures 5.1 and 5.2 show the viability of *L. acidophilus* L10 and *L. paracasei* L26 cultivated in skim milk containing different concentrations of inulin or Hi-maize. The initial cell concentration of *L. acidophilus* L10 and *L. paracasei* L26 on day 1 was significantly ($P < 0.05$) affected by the addition of prebiotics. Inulin and Hi-maize

supplementation likely resulted in improved growth of probiotics during fermentations prior to the storage. Moreover, the type of prebiotics also played a significant ($P < 0.05$) role on the probiotic growth, with inulin increasing the initial probiotic concentration by almost 1 log cycle in comparison to those obtained by Hi-maize (Figure 5.1). In general, the concentration of probiotics in all batches was reached and maintained above the lowest recommended therapeutic level of 6 log cfu/mL at the end of storage (Kurmann and Rasic, 1991). Although inulin did not enhance the viability of probiotic organisms during storage, it apparently had a significant ($P < 0.05$) effect on the initial growth performance of both probiotics and provided for a better retention of viability ($P > 0.05$) irrespective of its concentration during storage (Figures 5.1 and 5.2). On the contrary, the supplementation with Hi-maize was not as efficient in sustaining the viability of probiotics, which resulted in a significant ($P > 0.05$) decline in the concentration of probiotics towards the end of the storage period. The final cell concentration of probiotics in Hi-maize containing batches was similar ($P > 0.05$) to that in the prebiotic-free control product. This contrasted to a previous report by Bruno *et al*. (2002), who found that Hi-maize was the most effective in retention of viability of selected *Bifidobacterium* strains compared to inulin and raftilose. However, this discrepancy could be attributed to a strain dependant response of probiotics to supplemented prebiotics or the type of resistant starch and its degree of modification.

Similarly, there was a substantial improvement in the growth of yoghurt culture consisting of *S. thermophilus* St1342 and *L. delbrueckii* ssp. *bulgaricus* Lb1466 in the presence of inulin. Furthermore, inulin sustained the metabolic activity of the yoghurt culture better than hi-maize during cold storage, maintaining higher viable cell concentration as well as resulting in an increased production of primary metabolites, including lactic and acetic acids (Tables 5.2 and 5.3). Such a high concentration of organic acids had no detrimental effect on either culture in the presence of inulin, since the cells maintained fairly constant viability throughout storage.

Our results are consistent with previous reports, which showed a stimulatory effect of inulin on the growth of human fecal bacteria (Roberfroid *et al*., 1998), bifidobacteria (Akalin *et al*., 2004) and *Lactobacillus* strains (Desai *et al*., 2004). The mechanism by which inulin affected the probiotics in our culture could not be elucidated. Both cultures grew slow in prebiotic free yoghurt reaching therapeutic levels just above 7 log cfu/mL. The supplementation with prebiotics might have provided additional nutrients (Makras *et al*., 2005) or modified a negative environmental impact (Desai *et al*., 2004). Loss of viability of probiotic bacteria is typically more pronounced in fermented milk products due to the acid

injury to the organisms (Shah and Jelen, 1990; Shah, 2000b; Talwalkar and Kailasapathy, 2004).

5.3.2 Production of organic acids

The production of acetic and lactic acids by the individual cultures (*L. acidophilus* L10 and *L. paracasei* L26) increased ($P < 0.05$) in the presence of inulin and hi-maize compared to the control (Tables 5.2 and 5.3) in line with the growth stimulation. This was consistent with the findings of Desai *et al*. (2004), who found improved metabolic activity of several species of lactobacilli in the presence of selected prebiotics. Inulin created a better growth medium which stimulated the growth of *L. acidophilus* L10 and *L. paracasei* L26 better than hi-maize and resulted in higher ($P < 0.05$) production of acetic and lactic acids. Several authors reported that the utilization of prebiotics as well as the levels of primary metabolites varied depending on the strains (Bruno *et al*., 2002; Desai *et al*., 2004). The concentration of lactic acid clearly depended on the type of prebiotic, with inulin giving a higher ($P < 0.05$) concentration than hi-maize; however, the concentration of prebiotic had no effect ($P > 0.05$). Unlike the production of lactic acid, acetic acid was produced by *L. acidophilus* L10 and *L. paracasei* L26 in similar quantities ($P > 0.05$) in the presence of either prebiotic (Table 5.2). However, the amount of acetic acid in yoghurt samples with prebiotics was significantly ($P < 0.05$) higher than that of the control (Table 5.3). Although lactic acid production is desirable in fermented dairy foods, a high concentration of acetic acid may result in a distinct vinegary flavor.

The production of short-chain fatty acids (SCFA) such as butyrate and propionate by intestinal bacteria contributes towards host energy gain and may reduce the risk of colon cancer (Telang *et al*., 2005). However, the addition of inulin or hi-maize had no significant ($P > 0.05$) effect on the production of butyric acid and propionic acid by selected probiotic organisms (Tables 5.4 and 5.5). The concentration of propionic acid varied considerably in all yoghurt batches during 28 days of cold storage and no apparent trend was observed. The mixed yoghurt culture produced more ($P < 0.05$) propionic acid in the absence of probiotics (Table 5.4). This may indicate a possible utilization of this SCFA by probiotics. In general, the values obtained in our study agreed well with those reported by Fernandez-Garcia *et al*. (1998), who also observed no effect of fiber addition on propionic acid production by mixed yoghurt culture. The type of prebiotic appeared to influence the production of propionic acid by *L. acidophilus* L10 and *L. paracasei* L26. In our study with hi-maize supplemented at lower concentration (i.e. 0.5 or 1.0%), yielded more ($P < 0.05$) propionate than yoghurts

supplemented with higher concentrations of hi-maize. On the other hand, the concentration of butyric acid measured in all yoghurt samples with prebiotics was low and remained constant irrespective of the type or concentration of prebiotic, including that of the control without prebiotics (Table 5.5). This suggests that the addition of prebiotic may not influence the in situ production of butyric acid by the organisms studied. Fernandez-Garcia *et al*. (1998) observed the absence of butyric acid during the production of yoghurt fortified with fiber or sweetener.

5.3.3 Proteolytic activity of probiotic organisms in yoghurt

The production of yoghurt is a complex process involving many physical and chemical changes including proteolysis, which involves the progressive hydrolysis of the caseins to polypeptides, peptides and amino acids (Christensen *et al*., 1999; Shihata and Shah, 2000). The ability of LAB to grow to high cell densities in milk is dependent on a proteolytic system that can liberate essential amino acids from casein-derived peptides (Christensen *et al*., 1999). Proteolytic activity of selected probiotic strains, as well as yoghurt culture, in yoghurt supplemented with prebiotics during prolonged cold storage was estimated by determination of free amino groups using the OPA method (Table 5.6).

The results showed a significant ($P < 0.05$) improvement of proteolytic activity by probiotic organisms in presence of selected prebiotics. On the other hand, the proteolytic activity of studied strains was not significantly different ($P > 0.05$). In contrast to other metabolic activities, which were mainly governed by inulin addition, the proteolytic activity of *L. acidophilus* L10 and *L. paracasei* L26, each with yoghurt culture, was significantly ($P < 0.05$) improved in the presence of hi-maize, in comparison to that in the presence of inulin or without any supplementation. As expected, storage time played an important role in the extent of overall proteolytic activity. The GLM statistical analysis showed that the amount of liberated amino acids was significantly higher ($P < 0.05$) at 28 day of storage than that during initial stages. Although hi-maize improved proteolytic activity of probiotic strains substantially, it played no apparent role in sustaining the viability of cultures, since the cell concentrations declined substantially during storage of products containing hi-maize (Figures 5.1 and 5.2). In general, all organisms were proteolytic in yoghurt irrespective of prebiotic addition. This also confirmed previous finding that *L. acidophilus* L10 and *L. paracasei* L26 were highly proteolytic in prebiotic-free yoghurt (Section 3.4).

5.3.4 Viscosity of yoghurt

The viscosities of all batches of yoghurt with or without prebiotic supplementation are presented in Table 5.7. The statistical analysis showed a significant effect of organisms ($P < 0.0001$) and prebiotics ($P < 0.01$) on the viscosity of yoghurt. The addition of probiotic organisms, especially *L. paracasei* L26, resulted in a significantly ($P < 0.05$) higher viscosity compared to yoghurt with yoghurt culture alone. As shown in Table 5.7, the initial viscosity of yoghurt containing *L. paracasei* L26 (C2) was substantially higher than that containing *L. acidophilus* L10 (C3) or yoghurt culture alone (C1). This may have been caused by the production of exopolysaccharide (EPS), although this was not measured in this study. Evidently, the addition of inulin resulted in significantly higher ($P < 0.05$) viscosity in all batches of yoghurt over hi-maize and the control yoghurts. In this case, the viscosity even increased during storage, possibly due to increasing hydration (Bouzar *et al*., 1997). However, even though the increase in viscosity on addition of hi-maize was not as high as in the case of inulin, it was higher ($P = 0.05$) than that of the control, with a slight decline during storage (Table 5.7). Syrbe *et al*. (1998) reported the use of hydrocolloids in dairy formulations; such hydrocolloids physically stabilized a dispersed material and/or improved texture, resulting in high viscosity. Similar to our findings, higher viscosity values were observed by Fernandez-Garcia *et al*. (1998) in yoghurts containing fructose. The viscosity increase due to the addition of fiber has been attributed to interactions between these oligo- or polysaccharides and dairy proteins (Fernandez- Garcia *et al*., 1998; Syrbe *et al*., 1998; Sodini *et al*., 2002). In addition to the influence of prebiotics on viscosity, our study also showed a substantial effect of the probiotic organisms on viscosity.

5.4 CONCLUSIONS

The supplementation of yoghurt with prebiotics improved retention of viability of *L. acidophilus* L10 and *L. paracasei* L26 in yoghurt during cold storage, especially in the presence of inulin. Inulin was a better growth stimulant of probiotics than hi-maize. In addition, lower concentrations of inulin were found to be sufficient to stimulate the growth and retain the viability of the selected probiotic organisms and yoghurt culture in yoghurt in comparison to hi-maize. The presence of prebiotics did not influence the production of acetic acid or butyric acid by *L. acidophilus* L10 and *L. paracasei* L26. On the other hand, hi-maize, unlike inulin, at lower concentrations substantially influenced the production of propionic acid by the probiotic organisms. Furthermore, hi-maize improved the proteolytic

activity of the selected probiotic organisms. Addition of inulin and hi-maize to yoghurt increased viscosity in comparison to the control; however, supplementation with hi-maize slightly decreased the viscosity of yoghurt during storage at 4^{o}C, whereas inulin showed an increasing trend.

Table 5.1 The full factorial experimental design

Prebiotic	Concentration %	Culture		
		YC*	YC + La	YC + Lc
Hi-maize	0.5	YC + H# 0.5	YC + La H0.5	YC + Lc H0.5
	1.0	YC + H 1.0	YC + La H1.0	YC + Lc H1.0
	1.5	YC + H 1.5	YC + La H1.5	YC + Lc H1.5
Inulin	0.5	YC + Iβ 0.5	YC + La I0.5	YC + Lc I0.5
	1.0	YC + I 1.0	YC + La I1.0	YC + Lc I1.0
	1.5	YC + I 1.5	YC + La I1.5	YC + Lc I1.5
None		Control 1	Control 2	Control 3

YC* – yoghurt culture consisting of *L. delbrueckii* ssp. *bulgaricus* Lb1466 and *S. thermophilus* St1342; H# and Iβ – hi maize and inulin at concentrations of (0.5, 1.0 and 1.5)% (w/v), respectively; Control 1 = YC, Control 2 = YC + *L. paracasei*, Control 3 = YC + *L. acidophilus*, without any prebiotic.

Table 5.2 The concentration of lactic acid in yoghurt samples produced by yoghurt and probiotic cultures in the presence of different levels of selected prebiotics during 4-week cold storage

Culture	Prebiotics	Lactic acid, mg/mL				
		Period of storage, days				
		1	7	14	21	28
1. YC* + *L. acidophilus*	H#-0.5	13.63 Aa	13.63 Aa	16.36 Ab	16.38 Ab	16.00 Ab
	H-1.0	14.97 Aa	17.37 Aa	15.99 Aa	15.26 Aa	15.67 Aa
	H-1.5	14.72 Aa	14.84 Aa	15.68 Aa	15.23 Aa	15.52 Aa
	Iβ-0.5	16.04 Aa	17.18 Aa	19.11 Aa	18.35 Aa	19.28 Aa
	I-1.0	16.11 Aa	16.60 Aa	19.61 Aa	18.51 Aa	19.01 Aa
	I-1.5	15.99 Aa	15.69 Aa	17.95 Aa	18.22 Aa	18.70 Aa
	Cα3	15.04 Aa	13.89 Aa	16.12 Aa b	15.17 Aa	16.11 Aa b
2. YC + *L. casei*	H-0.5	13.83 Aa	14.20 Aa	15.67 Aa	15.18 Aa	15.15 Aa
	H-1.0	12.95 Aa	13.63 Aa	13.91 Aa	13.83 Aa	14.07 Aa
	H-1.5	13.91 Aa	14.25 Aa	17.25 Aa	15.35 ABCa	16.34 Aa
	I-0.5	15.56 Aa	16.79 Aa	19.08 Aa	17.27 ABCa	18.03 Aa
	I-1.0	16.26 Aa	17.47 Aa	18.05 Aa	18.16 BCDa	18.05 Aa
	I-1.5	16.86 Aa	15.36 Aa	16.90 Aa	19.58 BCDa	19.11 Ba
	C2	12.90 Aa	13.86 Aa	15.56 Aa	15.96 Aa	16.02 ABa
3. YC	H-0.5	11.81 Aa	10.88 Aa	13.31 Aab	14.12 Aab	14.33 Aab
	H-1.0	11.92 Aa	11.71 Aa	13.84 Aa	13.84 Aa	14.44 Aa
	H-1.5	11.83 Aa	11.75 Aa	13.12 Aa	13.34 Aa	13.96 Aa
	I-0.5	14.64 Aa	14.45 Aa	15.97 Aa	15.69 Aa	16.47 Aa
	I-1.0	14.73 Aa	16.39 Aa	19.85 ABa	16.47 Aa	17.87 Aa
	I-1.5	14.68 Aa	14.63 Aa	19.17 ABa	15.91 Aa	16.39 Aa
	C1	11.74 Aa	12.13 Aa	13.80 Aab	14.21 Aab	15.50 Ab
SEM		0.81				

YC* – yoghurt culture consisting of *L. delbrueckii* ssp. *bulgaricus* Lb1466 and *S. thermophilus* St1342; H# and Iβ – hi maize and inulin at concentrations of (0.5, 1.0 and 1.5)% (w/v), respectively; Cα = control; C1 = YC, C2 = YC + *L. paracasei,* C3 = YC + *L. acidophilus*, are controls without any prebiotic; SEM – pooled standard error of the mean for predetermined $P < 0.05$. ab Means in the same row with different small letter superscripts are significantly different; ABCD Means in the same column for a particular strain with different capital letter superscripts are significantly different.

Table 5.3 The concentration of acetic acid in yoghurt samples produced by yoghurt and probiotic cultures in the presence of different levels of selected prebiotics during 4-week cold storage

Culture	Prebiotics	Acetic acid, mg/mL				
		Period of storage, days				
		1	7	14	21	28
1. YC* + *L. acidophilus*	H#-0.5	6.82^{Aa}	5.53^{Aa}	7.40^{Aa}	6.55^{Aa}	6.55^{Aa}
	H-1.0	6.89^{Aa}	6.38^{Aa}	7.35^{Aa}	6.23^{Aa}	6.75^{Aa}
	H-1.5	6.80^{Aa}	5.58^{Aa}	5.84^{Aa}	5.65^{Aa}	6.40^{Aa}
	I$^{\beta}$-0.5	7.13^{Aa}	6.11^{Aa}	7.12^{Aa}	6.58^{Aa}	6.73^{Aa}
	I-1.0	6.96^{Aa}	5.71^{Aa}	7.33^{Aa}	6.71^{Aa}	7.10^{Aa}
	I-1.5	6.52^{Aa}	6.36^{Aa}	7.50^{Aa}	6.30^{Aa}	7.09^{Aa}
	C$^{\alpha}$3	6.27^{Aa}	4.32^{Aa}	6.98^{Aa}	4.86^{Aa}	5.51^{Aa}
2. YC + *L. casei*	H-0.5	6.86^{Aa}	5.38^{Aa}	6.64^{Aa}	6.18^{Aa}	6.81^{Aa}
	H-1.0	6.78^{Aa}	5.54^{Aa}	5.87^{Aa}	5.92^{Aa}	6.48^{Aa}
	H-1.5	6.16^{Aa}	5.63^{Aa}	6.92^{Aa}	6.34^{Aa}	7.08^{Aa}
	I-0.5	6.99^{Aa}	5.82^{Aa}	7.74^{Aa}	6.58^{Aa}	7.52^{Aa}
	I-1.0	7.03^{Aa}	6.35^{Aa}	7.24^{Aa}	6.87^{Aa}	6.74^{Aa}
	I-1.5	6.89^{Aa}	5.48^{Aa}	6.43^{Aa}	6.94^{Aa}	7.72^{Aa}
	C2	5.60^{Aa}	4.82^{Aa}	5.15^{Aa}	4.73^{Aa}	4.72^{Aa}
3. YC	H-0.5	6.56^{Aa}	5.01^{Aa}	6.85^{Aa}	6.69^{Aa}	6.67^{Aa}
	H-1.0	6.83^{Aa}	5.59^{Aa}	7.24^{Aa}	6.37^{Aa}	6.70^{Aa}
	H-1.5	6.64^{Aa}	5.36^{Aa}	7.23^{Aa}	6.08^{Aa}	6.49^{Aa}
	I-0.5	6.87^{Aa}	5.73^{Aa}	7.36^{Aa}	6.47^{Aa}	6.81^{Aa}
	I-1.0	6.71^{Aa}	5.97^{Aa}	7.79^{Aa}	6.39^{Aa}	6.66^{Aa}
	I-1.5	6.65^{Aa}	5.24^{Aa}	7.44^{Aa}	6.49^{Aa}	6.49^{Aa}
	C1	6.68^{Aa}	5.03^{Aa}	6.49^{Aa}	4.46^{Aa}	6.01^{Aa}
SEM		0.40				

YC* – yoghurt culture consisting of *L. delbrueckii* ssp. *bulgaricus* Lb1466 and *S. thermophilus* St1342; H# and I$^{\beta}$ – hi maize and inulin at concentrations of (0.5, 1.0 and 1.5)% (w/v), respectively; C$^{\alpha}$ = control; C1 = YC, C2 = YC + *L. paracasei,* C3 = YC + *L. acidophilus*, are controls without any prebiotic; SEM – pooled standard error of the mean for predetermined $P < 0.05$. ^{ab} Means in the same row with different small letter superscripts are significantly different; ^{AB} Means in the same column for a particular strain with different capital letter superscripts are significantly different.

Table 5.4 The concentration of propionic acid in yoghurt samples produced by yoghurt and probiotic cultures in the presence of different levels of selected prebiotics during 4-week cold storage

Culture	Prebiotics	Propionic acid, μg/mL				
		Period of storage, days				
		1	7	14	21	28
1. YC* + *L. acidophilus*	H#-0.5	34.92^{Aa}	38.00^{Aa}	60.31^{Aa}	46.81^{Aa}	55.85^{Aa}
	H-1.0	30.80^{Aa}	49.53^{Aa}	69.50^{Aa}	49.99^{Aa}	62.43^{Aa}
	H-1.5	27.49^{Aa}	38.93^{Aa}	31.60^{Aa}	38.15^{Aa}	44.27^{Aa}
	I$^{\beta}$-0.5	26.52^{Aa}	30.49^{Aa}	37.77^{Aa}	24.45^{Aa}	37.93^{Aa}
	I-1.0	27.79^{Aa}	30.31^{Aa}	40.95^{Aa}	35.02^{Aa}	47.95^{Aa}
	I-1.5	28.70^{Aa}	33.08^{Aa}	40.41^{Aa}	34.63^{Aa}	31.49^{Aa}
	C$^{\alpha}$3	31.69A^{a}	20.94a^{a}	38.88A^{a}	16.88a^{a}	29.34^{ABa}
2. YC + *L. casei*	H-0.5	28.08^{Aa}	33.14^{Aa}	52.56^{Aa}	38.70^{Aa}	49.87^{Aa}
	H-1.0	42.44^{Aa}	39.27^{Aa}	55.41^{Aa}	50.17^{Aa}	57.63^{Aa}
	H-1.5	25.05^{Aa}	24.68^{Aa}	35.95^{Aa}	25.01^{Aa}	31.17^{Aa}
	I-0.5	36.47^{Aa}	35.49^{Aa}	41.21^{Aa}	29.55^{Aa}	42.70^{Aa}
	I-1.0	25.03^{Aa}	24.14^{Aa}	45.79^{Aa}	41.77^{Aa}	41.79^{Aa}
	I-1.5	24.45^{Aa}	24.60^{Aa}	38.91^{Aa}	36.77^{Aa}	35.86^{Aa}
	C2	20.93^{Aa}	19.00^{Aa}	15.02^{Aa}	42.74^{Aa}	32.40^{Aa}
3. YC	H-0.5	46.69^{Aa}	41.35^{Aa}	98.82^{Aa}	60.79^{Aa}	57.51^{Aa}
	H-1.0	35.86^{Aa}	40.06^{Aa}	102.30^{Aa}	49.25^{Aa}	79.94^{Aa}
	H-1.5	55.13^{Aa}	41.05^{Aa}	73.10^{Aa}	64.50^{Aa}	68.00^{Aa}
	I-0.5	30.47^{Aa}	34.66^{Aa}	54.90^{Aa}	34.50^{Aa}	54.14^{Aa}
	I-1.0	28.14^{Aa}	26.49^{Aa}	57.53^{Aa}	30.41^{Aa}	43.83^{Aa}
	I-1.5	32.07^{Aa}	21.68^{Aa}	56.32^{Aa}	29.89^{Aa}	54.15^{Aa}
	C1	58.83^{Aa}	34.41^{Aa}	77.62^{Aa}	93.10^{Aa}	66.69^{Aa}
SEM		10.86				

YC* – yoghurt culture consisting of *L. delbrueckii* ssp. *bulgaricus* Lb1466 and *S. thermophilus* St1342; H# and I$^{\beta}$ – hi maize and inulin at concentrations of (0.5, 1.0 and 1.5)% (w/v), respectively; C$^{\alpha}$ = control; C1 = YC, C2 = YC + *L. paracasei,* C3 = YC + *L. acidophilus*, are controls without any prebiotic; SEM – pooled standard error of the mean for predetermined $P < 0.05$. ^{ab} Means in the same row with different small letter superscripts are significantly different; ^{AB} Means in the same column for a particular strain with different capital letter superscripts are significantly different.

Table 5.5 The concentration of butyric acid in yoghurt samples produced by yoghurt and probiotic cultures in the presence of different levels of selected prebiotics during 4-week cold storage

Culture	Prebiotics	Butyric acid, mg/mL Period of storage, days				
		1	7	14	21	28
1. YC[*] + *L. acidophilus*	H[#]-0.5	3.91^{Aa}	3.42^{Aa}	3.45^{Aa}	3.87^{Aa}	3.87^{Aa}
	H-1.0	3.80^{Aa}	3.43^{Aa}	3.63^{Aa}	3.81^{Aa}	3.52^{Aa}
	H-1.5	3.85^{Aa}	3.61^{Aa}	3.71^{Aa}	3.97^{Aa}	3.67^{Aa}
	I[β]-0.5	3.83^{Aa}	3.71^{Aa}	3.78^{Aa}	3.87^{Aa}	3.92^{Aa}
	I-1.0	3.58^{Aa}	3.53^{Aa}	3.48^{Aa}	3.88^{Aa}	3.75^{Aa}
	I-1.5	3.74^{Aa}	3.48^{Aa}	3.44^{Aa}	3.81^{Aa}	3.64^{Aa}
	C[α]3	4.05^{Aa}	3.94^{Aa}	3.69^{Aa}	4.22^{Aa}	3.78^{Aa}
2. YC + *L. casei*	H-0.5	4.11^{Aa}	3.89^{Aa}	3.67^{Aa}	3.98^{Aa}	3.66^{Aa}
	H-1.0	3.54^{Aa}	3.38^{Aa}	3.14^{Aa}	3.46^{Aa}	3.78^{Aa}
	H-1.5	4.02^{Aa}	3.68^{Aa}	3.81^{Aa}	4.24^{Aa}	3.90^{Aa}
	I-0.5	3.72^{Aa}	3.38^{Aa}	3.53^{Aa}	3.63^{Aa}	3.64^{Aa}
	I-1.0	3.89^{Aa}	3.81^{Aa}	3.22^{Aa}	3.75^{Aa}	3.77^{Aa}
	I-1.5	3.68^{Aa}	3.49^{Aa}	3.28^{Aa}	3.96^{Aa}	3.62^{Aa}
	C2	3.60^{Aa}	3.95^{Aa}	4.13^{Aa}	4.14^{Aa}	3.52^{Aa}
3. YC	H-0.5	3.57^{Aa}	2.90^{Aa}	3.16^{Aa}	3.64^{Aa}	3.79^{Aa}
	H-1.0	3.57^{Aa}	3.68^{Aa}	3.65^{Aa}	4.09^{Aa}	3.53^{Aa}
	H-1.5	3.68^{Aa}	3.75^{Aa}	3.28^{Aa}	3.98^{Aa}	3.87^{Aa}
	I-0.5	3.41^{Aa}	3.21^{Aa}	3.21^{Aa}	3.26^{Aa}	3.44^{Aa}
	I-1.0	3.49^{Aa}	3.40^{Aa}	3.28^{Aa}	3.45^{Aa}	3.63^{Aa}
	I-1.5	3.38^{Aa}	3.19^{Aa}	3.19^{Aa}	3.29^{Aa}	3.10^{Aa}
	C1	3.82^{Aa}	3.06^{Aa}	2.87^{Aa}	3.51^{Aa}	2.91^{Aa}
SEM		0.20				

YC[*] – yoghurt culture consisting of *L. delbrueckii* ssp. *bulgaricus* Lb1466 and *S. thermophilus* St1342; H[#] and I[β] – hi maize and inulin at concentrations of (0.5, 1.0 and 1.5)% (w/v), respectively; C[α] = control; C1 = YC, C2 = YC + *L. paracasei*, C3 = YC + *L. acidophilus*, are controls without any prebiotic; SEM – pooled standard error of the mean for predetermined $P < 0.05$. [ab] Means in the same row with different small letter superscripts are significantly different; [AB] Means in the same column for a particular strain with different capital letter superscripts are significantly different.

Table 5.6 Proteolytic activity of yoghurt and probiotic cultures in yoghurt samples prepared by addition of selected prebiotics and stored for 4 weeks at 4°C

Culture	Prebiotics	Proteolytic activity, absorbance$_{340}$				
		Period of storage, days				
		1	7	14	21	28
1. YC* + *L. acidophilus*	H$^{\#}$-0.5	0.93 ^{Aa}	0.96 ^{Aa}	0.89 ^{Aa}	0.91 ^{Aa}	0.84 ^{Aa}
	H-1.0	0.90 ^{Aa}	0.81 ^{Aa}	0.80 ^{Aa}	0.81 ^{Aa}	0.96 ^{Aa}
	H-1.5	0.74 ^{Aa}	0.81 ^{Aa}	1.01 ^{Aa}	0.70 ^{Aab}	0.70 ^{ABCab}
	I$^{\beta}$-0.5	0.60 ^{ABa}	0.47 ^{Ba}	0.68 ^{ACa}	0.86 ^{Aab}	0.57 ^{BCa}
	I-1.0	0.59 ^{ABa}	0.74 ^{Aa}	0.73 ^{ACab}	0.81 ^{Aab}	0.54 ^{BCac}
	I-1.5	0.70 ^{ABa}	0.56 ^{ABa}	0.70 ^{ABCab}	0.77 ^{Aab}	0.67 ^{ACa}
	C$^{\alpha}$3	0.80 ^{Aa}	0.77^{Aa}	0.45 ^{BCb}	0.57 ^{Aab}	1.03 ^{ADa}
2. YC + *L. casei*	H-0.5	1.03 ^{Aa}	0.86 ^{Aa}	0.81 ^{Aa}	0.87 ^{Aa}	1.02 ^{Aa}
	H-1.0	0.99 ^{ABa}	0.99 ^{Aa}	0.88 ^{Aab}	0.75 ^{Aa}	1.09 ^{Aa}
	H-1.5	0.87 ^{Ba}	0.76 ^{ABab}	0.84 ^{Aab}	0.63 ^{Ab}	0.55 ^{Bb}
	I-0.5	0.72 ^{BCa}	0.59 ^{ABa}	0.61 ^{Aa}	0.72 ^{Aa}	0.64 ^{Ba}
	I-1.0	0.71 ^{BCa}	0.64 ^{ABa}	0.75 ^{Aa}	0.59 ^{Aa}	0.76 ^{ABa}
	I-1.5	0.64 ^{BCa}	0.72 ^{Aa}	0.72 ^{Aa}	0.30 ^{BCba}	0.48 ^{Ba}
	C2	0.70 ^{BCa}	0.63 ^{ABa}	0.56 ^{ABa}	0.73 ^{Aa}	0.75 ^{ABCa}
3. YC	H-0.5	0.86 ^{Aa}	0.80 ^{Aa}	0.89 ^{Aa}	0.56 ^{Aab}	0.89 ^{Aa}
	H-1.0	0.96 ^{Aa}	0.78 ^{Aa}	0.88 ^{Aa}	0.88 ^{ABa}	0.92 ^{Aa}
	H-1.5	1.02 ^{Aa}	0.84 ^{Aa}	1.10 ^{Aa}	1.09 ^{Ba}	0.93 ^{Aa}
	I-0.5	0.75 ^{Aa}	0.55 ^{ACa}	0.69 ^{ABa}	1.35 ^{Bb}	0.57 ^{Aa}
	I-1.0	0.81 ^{Aa}	0.61 ^{Aa}	0.77 ^{Aa}	0.57 ^{Aa}	0.48 ^{Aa}
	I-1.5	0.75 ^{Aa}	1.10 ^{Bb}	0.75 ^{Aa}	0.77 ^{ABa}	0.51 ^{Aa}
	C1	0.88 ^{Aa}	0.72 ^{Aa}	0.71 ^{ACa}	0.96 ^{ABa}	0.87 ^{Aa}
SEM		0.06				

YC* – yoghurt culture consisting of *L. delbrueckii* ssp. *bulgaricus* Lb1466 and *S. thermophilus* St1342; H$^{\#}$ and I$^{\beta}$ – hi maize and inulin at concentrations of (0.5, 1.0 and 1.5)% (w/v), respectively; C$^{\alpha}$ = control; C1 = YC, C2 = YC + *L. paracasei,* C3 = YC + *L. acidophilus*, are controls without any prebiotic; SEM – pooled standard error of the mean for predetermined $P < 0.05$. ^{abc} Means in the same row with different small letter superscripts are significantly different; ^{ABC} Means in the same column for a particular strain with different capital letter superscripts are significantly different.

Table 5.7 Apparent viscosity of yoghurt samples prepared by yoghurt and probiotic cultures with supplementation of different concentrations of selected prebiotics during prolonged cold storage for 28 days

Culture	Prebiotic	Viscosity, Pa s					SEM
		Storage period, days					
		1	7	14	21	28	
1. YC* + *L. acidophilus*	H#-0.5	69.85^{Aa}	57.37^{Ab}	53.06^{Ab}	54.09^{Ab}	51.77^{Ab}	3.97
	H-1.0	65.52^{ABa}	72.57^{Bb}	70.15^{Bab}	68.55^{Bab}	60.06^{Bc}	
	H-1.5	72.40^{Aa}	69.43^{Ba}	69.01^{Bb}	63.94^{Bc}	54.37^{ABd}	
	Iβ-0.5	63.38^{Ba}	60.66^{Aa}	67.18^{Bab}	72.66^{BCb}	83.39^{Dc}	
	I-1.0	65.71$^{AB\ a}$	71.11^{Bab}	76.79^{Bb}	72.71^{BCab}	69.15^{Cab}	
	I-1.5	79.52^{Ca}	83.47^{Ca}	78.39^{Ba}	79.63^{Ca}	82.05^{Da}	
	Cα3	49.64^{Da}	50.37^{Aa}	57.36^{Aa}	57.11^{Aa}	55.10^{ABa}	5.61
2. YC + *L. casei*	H-0.5	53.45^{Aa}	63.49^{ABb}	60.77^{Aab}	62.82^{Aab}	55.39^{Aab}	3.97
	H-1.0	61.59^{Ba}	59.24^{Aa}	55.72^{Aa}	58.28^{Aa}	56.60^{Aa}	
	H-1.5	65.29^{Ba}	64.44^{ABa}	60.05^{Aa}	63.93^{Aa}	64.81^{Ba}	
	I-0.5	64.99^{Ba}	70.19^{Bab}	72.19^{Bab}	75.84^{Bb}	74.83^{Cb}	
	I-1.0	69.64^{Ba}	68.51^{ABCa}	69.39^{Ba}	73.54^{Ba}	75.33^{Ca}	
	I-1.5	63.68^{Ba}	75.65^{Cb}	70.49^{Bab}	71.96^{Bb}	72.24^{BCb}	
	C2	61.68^{Ba}	66.30^{ABab}	73.93^{Bb}	73.01^{Bb}	71.62^{BCab}	5.61
3. YC	H-0.5	48.86^{Ba}	39.89^{Ab}	34.31^{Ab}	38.37^{Ab}	39.63^{Ab}	3.97
	H-1.0	59.16^{Ca}	43.10^{Ab}	42.26^{ABb}	47.21^{ABb}	47.61^{ABb}	
	H-1.5	47.58^{Ba}	58.53^{Bb}	52.93^{Bab}	47.04^{ABb}	44.86^{ABb}	
	I-0.5	50.19^{Ba}	52.57^{ABa}	49.39^{Ba}	53.18^{Ba}	52.84^{Ba}	
	I-1.0	45.02^{Ba}	54.92^{Bb}	52.16^{Bab}	52.54^{Bab}	51.11^{Bab}	
	I-1.5	50.06^{Ba}	50.44^{ABa}	49.88^{Ba}	50.87^{Ba}	53.68^{Ba}	
	C1	30.45^{Aa}	42.79^{Ab}	48.41^{Bb}	41.69^{Aab}	37.84^{Aab}	5.61

YC* – yoghurt culture consisting of *L. delbrueckii* ssp. *bulgaricus* Lb1466 and *S. thermophilus* St1342; H# and Iβ – hi maize and inulin at concentrations of (0.5, 1.0 and 1.5)% (w/v), respectively; Cα = control; C1 = YC, C2 = YC + *L. paracasei,* C3 = YC + *L. acidophilus*, are controls without any prebiotic; SEM – pooled standard error of the mean for predetermined $P < 0.05$. ^{abcd} Means in the same row with different small letter superscripts are significantly different; ^{ABCD} Means in the same column for a particular strain with different capital letter superscripts are significantly different.

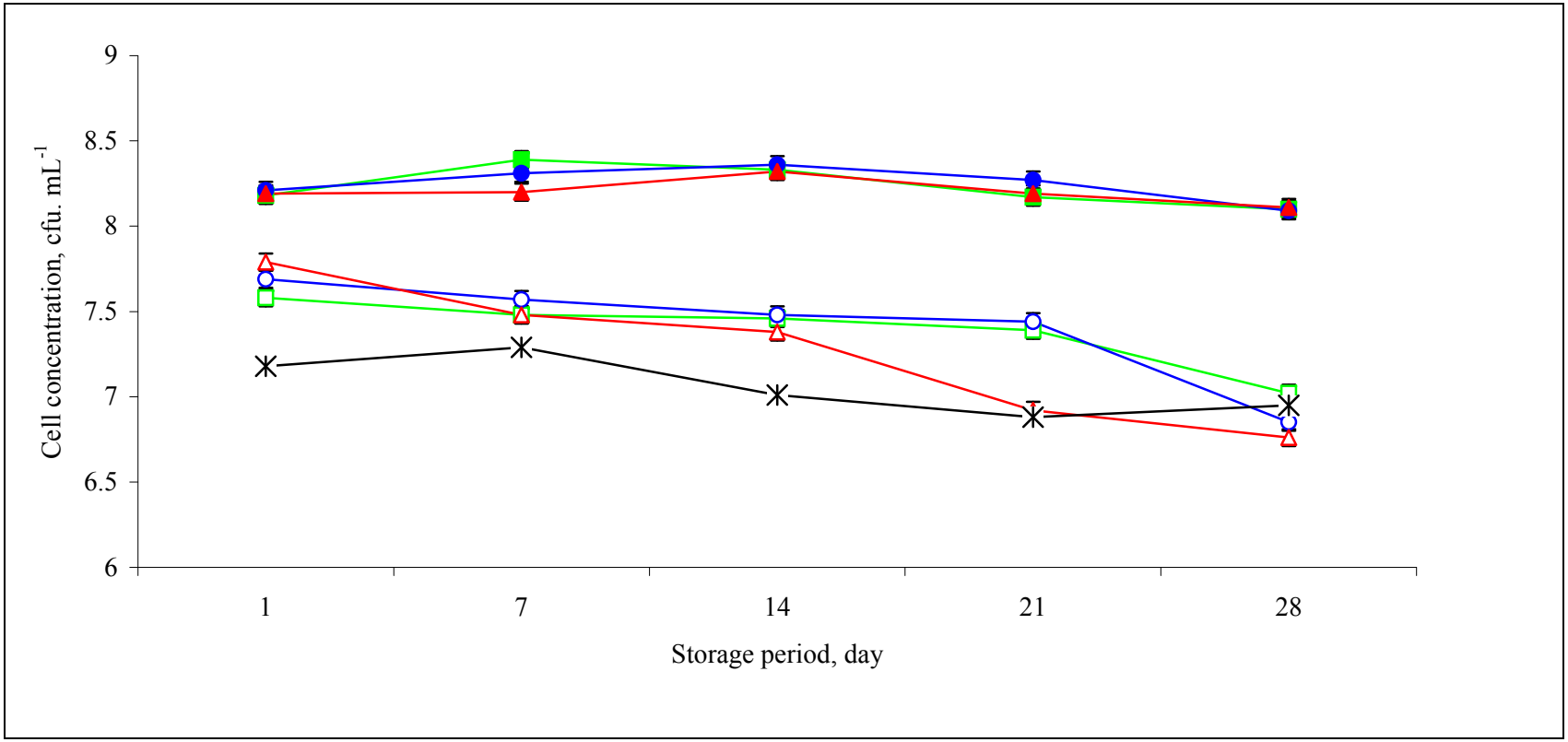

Figure 5.1 The viability of *L. acidophilus* L10 in yoghurt samples produced by yoghurt culture (*S. thermophilus* St1342, *L. delbrueckii* ssp. *bulgaricus* Lb 1466) in the presence of different levels of selected prebiotics (hi-maize and inulin) during 4 weeks of storage at 4°C (Error bars represent a pooled standard error of the mean, SEM = 0.05 cfu/mL). Open symbols, H = hi-maize and closed symbols, I = inulin at concentrations of 0.5, 1.0 or 1.5% (w/v), respectively; a star, C = control without any prebiotic. (□) H-0.5, (○) H-1.0, (Δ) H-1.5; (■) I-0.5, (●) I-1.0, (▲) I-1.5, (*) C

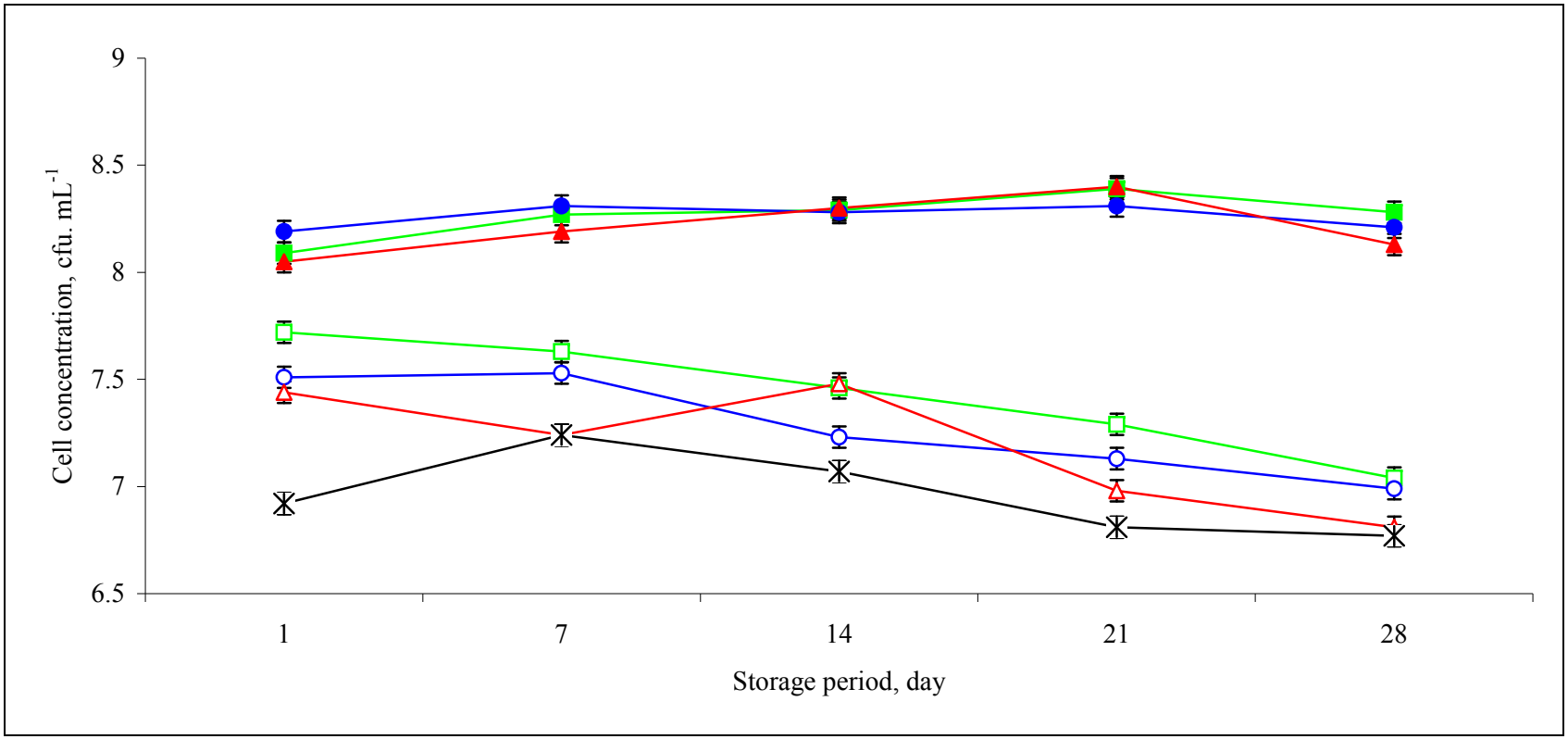

Figure 5.2 The viability of *L. casei* L26 in yoghurt samples produced by yoghurt culture (*S. thermophilus* St1342, *L. delbrueckii* ssp. *bulgaricus* Lb 1466) in the presence of different levels of selected prebiotics (hi-maize and inulin) during 4-week cold storage (Error bars represent a pooled standard error of the mean, SEM = 0.05 cfu/mL). Open symbols, H = hi-maize and closed symbols, I = inulin at concentrations of 0.5, 1.0 or 1.5% (w/v), respectively; a star, C = control without any prebiotic. (□) H-0.5, (○) H-1.0, (Δ) H-1.5; (■) I-0.5, (●) I-1.0, (▲) I-1.5, (⁎) C

6.0 Proteolytic activity of dairy lactic acid bacteria and probiotics as determinant of viability and *in vitro* angiotensin-converting enzyme inhibitory activity in fermented milk

6.1 INTRODUCTION

Incorporation of probiotic organisms such as *Lactobacillus acidophilus, Bifidobacterium* sp., and *L. casei* in fermented dairy products provides a potential to improve health status of consumers. A growing public awareness of diet related health issues and mounting evidence regarding health benefits of probiotics have increased consumers demand for foods containing probiotic organisms. Probiotics are defined by the Food and Agricultural Organization of the United Nations (FAO) and World Health Organization (WHO) as "live microorganisms, which when administered in adequate amounts confer a health benefit on the host." (FAO/WHO, 2002). A number of health benefits of probiotic organisms has been suggested and described in numerous reviews (Lourens-Hattingh and Viljoen, 2001; Shah, 2000a).

However, certain criteria during production of probiotic foods need to be satisfied to ensure maximization of therapeutic properties of probiotics. These include the incorporation of sufficient numbers of probiotic microorganisms into the product, maintenance of viable populations during shelf life of the food and subsequent survival of these organisms through the gastrointestinal tract (GIT) (Ravula and Shah, 1998; Ziemer and Gibson, 1998). Fermented dairy foods present ideal delivery systems for probiotics to the human GIT, since they may provide a favourable environment, which promotes growth and enhances viability of these organisms (Lourens-Hattingh and Viljoen, 2001). On the other hand, several factors such as low pH, presence of hydrogen peroxide and dissolved oxygen as well as the state of culturing and storage conditions may affect the survival of probiotic bacteria in fermented dairy products (Shah, 2000b; Shah and Ravula, 2000; Talwalkar and Kailasapathy, 2004).

Lactic acid bacteria (LAB) including probiotic organisms are fastidious in nature, requiring numerous essential growth factors. Milk, although a rich growth medium, contains low concentration of free amino acids and peptides to efficiently support growth of LAB (Shihata and Shah, 2000; Vasiljevic *et al*., 2005). In response to this limitation, LAB have developed a complex system of proteinases and peptidases, which enable them to utilise casein as an additional source of organic nitrogen (Smid *et al*., 1991). On the other hand, Klaver *et al*. (1993) reported that *Bifidobacterium* strains were not as proteolytic as other

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LAB. This may explain why *Bifidobacterium* spp. grows slowly in milk and may require supplementation of peptides and amino acids from external sources (Dave and Shah, 1998).

The proteolytic activities of LAB including yoghurt starter bacteria and probiotic organisms have been studied extensively and proteolytic enzymes have been isolated and characterised (Law and Haandrikman, 1997; Shihata and Shah, 2000; Wohlrab and Bockelmann, 1992). Such an interest has been derived from the importance of proteolytic system of LAB in cheese ripening and rapid growth in milk during fermentation as well as improved survival during storage (Chapter 3). This ability of dairy LAB and probiotics has become even more important upon realising that a range of bioactive peptides may be liberated due to microbial action. Biologically active peptides are generated during milk fermentation by proteolytic enzymes produced by various LAB such as *L. helveticus, L. lactis* subsp. *cremoris* FT4 and *L. delbrueckii* ssp. *bulgaricus* SS1 (Gobbetti *et al*., 2002; Nakamura *et al*., 1995). These biologically active peptides include hypotensive peptides which inhibit angiotensin-I-converting enzyme (ACE), opioid agonist and antagonist peptides, and mineral binding, immunomodulatory, antibacterial, and antithrombotic peptides (Pihlanto and Korhonen, 2003; Shah, 2000c). Angiotensin I-converting enzyme regulates blood pressure via formation of vasopressor angiotensin II from angiotensin I (Skeggs *et al*., 1956). Inhibition of ACE mainly results in an overall hypotensive effect. High proteolytic activity is expected to promote good cell growth and ACE-inhibitory activity in fermented milk. As stated, the proteolytic activity of dairy cultures is very important governing factor playing a major role in various cellular and physiological processes.

The aims of our study were to assess the proteolytic activity of selected dairy lactic acid bacteria and probiotic organisms as determinant of growth and in vitro ACE inhibitory activity in fermented milk.

6.2 MATERIALS AND METHODS

6.2.1 Bacterial Cultures

L. acidophilus LAFTI® L10, *B. lactis* LAFTI® B94 and *L. casei* LAFTI® L26 were obtained from DSM Food Specialties (Moorebank, NSW, Australia) and have been reported to have probiotic properties (Crittenden *et al*., 2005). *S. thermophilus* St1342, *L. delbrueckii* subsp. *bulgaricus* Lb 1466, *L. acidophilus* La 4962, *B. longum* Bl 536 and *L. casei* Lc 279 were supplied by the Victoria University Culture Collection (Werribee, Australia). Each strain was propagated in de Mann Rogosa Sharpe (MRS) broth (Oxoid, West Heidelberg,

Australia) at 37°C with the exception of *L. delbrueckii* ssp. *bulgaricus* Lb 1466 which was propagated at 42°C. For propagation of *Bifidobacterium*, sterile MRS broth was supplemented with 0.05% L-cysteine.hydrochloride to provide anaerobic condition and stimulate their growth (Shah and Ravula 2000). After three successive transfers of 20 h incubation each, the activated organisms were used for the preparation of the pre-inocula for further experiments. The pre-inocula were prepared by transferring 1% (v/v) of activated culture to 10 mL aliquots of reconstituted skim milk (RSM) supplemented with 2% glucose and 1% yeast extract.

6.2.2 Proteolytic systems of LAB

6.2.2.1 Preparation of intracellular and cell wall extracts

Individual cultures of *L. acidophilus* L10, *Bifidobacterium* B94, *L. casei* L26, *S. thermophilus* St1342, *L. delbrueckii* ssp. *bulgaricus* Lb 1466*, L. acidophilus* La 4962, *Bifidobacterium* Bl 536 and *L. casei* Lc 279 were propagated three successive times in RSM and finally in MRS broth according to the methods previously reported (Shihata and Shah, 2000; Wohlrab and Bockelmann, 1992). The latter was performed to prevent a carryover of milk proteins and their interference with the assay. Thus after two subculturing in MRS medium for 18 h at 37°C, cultures were cultivated separately in 100 mL batches of MRS broth at 42°C. The cells were then harvested by centrifugation at 4,000 x g at 4°C for 30 min at the end of the logarithmic growth phase at approximately 20 h which was determined by measuring the absorbance at 600 nm. The supernatant was designated as the cell-free extracellular enzymatic extract (EE). The cell pellet obtained was washed twice with 0.9% (w/v) NaCl solution and resuspended in 10% volume of original growth medium with 0.05 M Tris-HCl buffer, pH 8.5. The resulting cell dispersion was sonicated for 5 min at 30 s intervals at 4°C. The supernatant obtained after centrifugation at 4,000 x g for 30 min at 4°C was designated as the intracellular enzymatic extract (IE).

6.2.2.2 Determination of protein concentration

The protein content of the EE and IE extracts was estimated using the method of Bradford (Bradford, 1976). A 0.1 mL aliquot of the enzyme solution and 3 mL aliquot of the Bradford reagent (Sigma) were vortexed gently to mix thoroughly and the samples were incubated at room temperature for 30 min after which the absorbance was measured at 595 nm. Bovine serum albumin (Sigma) was used as a standard. The protein concentration of the

samples was determined by comparing the net absorbance values obtained at 595 nm against the standard curve.

6.2.2.3 Thin-layer chromatography and enzyme assay

Endopeptidase and tripeptidase activities of the EE and IE extracts were detected by thin-layer chromatography (TLC) according to the method of Shihata and Shah (2000) and Tan and Konings (1990) with some modifications. Each reaction mixture contained 60 µL of a 2 mM substrate (Gly-Ala-Tyr, Gly-Leu-Phe and Bradykinin) in 20 mM Tris-HCl pH 7.0 and 20 µL of extract. The reaction mixture was incubated for 60 min at 37^{o}C and was stopped by adding 10 µL of 30% acetic acid and cooling to 4^{o}C. Ten microlitres of the mixture were spotted onto a precoated silica gel 60 plate (50 x 100 x 0.25) mm (Alltech Associates Pty. Ltd., Baulkham Hills, Australia). A 4:1:1 (v/v/v) mixture of *n*-butanol:acetic acid:water was used as the mobile phase. As a control, 2 mM of each standard peptide was also spotted onto the plate. Silica gels were stained by spraying with 0.1% (w/v) ninhydrin in 99% ethanol. Peptides and amino acids became visible after incubating the plate in an oven for 5 min at 80^{o}C.

The proteolytic strains of probiotic and yoghurt bacteria were assessed for selected peptidolytic activities using Pro-Ile, Leu-Tyr, Leu-Gly, Ala-Met, and Ala-His as well as Gly-Ala-Tyr, Gly-Leu-Phe and bradykinin as substrates according to the method of Shihata and Shah (2000) and Wohlrab and Bockelmann (1992). The reaction mixture contained 10 µL of enzyme solution, 424 µL of 50 mM Tris-HCl pH 7.5, 50 µL of 22 mM substrate, 25 µL of peroxidase (5 mg/mL in 0.8 M $(NH_4)_2SO_4$), 25 µl of L-amino acid oxidase (2 mg/mL in distilled water) and 25 µL of *o*-dianisidine (11.5 mM). The reaction mixture was incubated at 50^{o}C for 20 min and was stopped by the addition of 50 µL of dithiothreitol (120 mM). Oxidation of *o*-dianisidine coupled to substrate hydrolysis resulted in an increase in brown colour which was measured at 436 nm. Enzyme activity was calculated using a molar absorbance coefficient of 8100/mol/cm and defined as the amount of enzyme required to oxidise 1 µmol of o-dianisidine per min under assay conditions (Wohlrab and Bockelmann, 1992). The specific activity was expressed as units per milligram of protein.

6.2.2.4 Assessment of X-prolyl-dipeptidyl aminopeptidase activity

X-prolyl-dipeptidyl aminopeptidase (PepX) activity of dairy cultures is a very important characteristic due to high proline content in milk protein. This activity was assayed as described previously (Pan *et al*., 2005) with glycyl-prolyl p-nitroanilide (Sigma)

(Gly-Pro-pNA) as the substrate with some modifications. The incubation mixture contained 50 µL of 6.4 mM of substrate, 2.85 mL of 50 mM Tris-HCl buffer pH 7.0, and 100 µL of cell-free intracellular extract (IE) in 50 mM Tris-HCl buffer pH 7.0. The mixture was incubated at 37^{o}C for 20 min and the reaction was stopped by adding 500 µL of 30% acetic acid. The extent of hydrolysis was measured with the Cary IE UV/visible spectrophotometer (Varian Australia Pty. Ltd., Melbourne, Australia) at 410 nm. The same experiment was also performed with cell-free extracellular (EE) enzymatic extracts. One unit of enzyme activity was defined as the amount of enzyme required to release 1 µmol of *p*-nitroanilide per min under assay conditions. The enzyme activity reported is expressed as specific Pep X activity defined as units of enzyme activity per milligram of proteins. A unit of activity is derived from the enzyme content required to liberate 1 µmol of p-nitroanilide per min under assay conditions.

6.2.3 Preparation of fermented milk

Individually activated cultures of *L. acidophilus* (L10 and La 4962), *Bifidobacterium* spp. (*B. lactis* B 94 and *B. longum* Bl 536), *L. casei* (L26 and Lc 279), *S. thermophilus* (St 1342) and *L. delbrueckii* ssp. *bulgaricus* (Lb 1466) in the form of pre-inoculate (section 2.1) were used for milk fermentations. Each organism was transferred into sterile RSM (12%) to obtain approximately 10^{8} cfu/mL. The inoculated milk was incubated at 37^{o}C for 20 h, and this step was repeated twice to prevent any carry overs (i.e. yeast extract) that would likely interfere with the assays (Leclerc *et al*., 2002). Batch fermentations were further carried out with each culture in 12% (w/w) RSM. Eight batches of 250 mL aliquots of RSM were aseptically prepared with 1% (v/v) of each of the cultures and incubated at 42^{o}C for 24 h. The fermentation experiments were performed in triplicate. A control consisted of uninoculated RSM. During fermentation, aliquots of each batch were taken at 0, 3, 6, 9, 12 and 24 h to monitor cell growth, organic acid production and pH changes. Proteolysis was monitored at 0, 6, 12 and 24 h.

6.2.4 Cell growth

The methods of Leclerc *et al*. (2002) and Ravula and Shah (1998) were used to assess the cell growth. One gram of each batch, aseptically sampled at 0, 3, 6, 9, and 24 h, was 10-fold serially diluted (10^{3} to 10^{7}) in 0.15% sterile peptone (Oxoid) and water diluent. Enumeration of the bacteria was performed on MRS agar (Amyl media, Dandenong, Australia) using the pour plate technique as described previously (Dave and Shah, 1996;

Tharmaraj and Shah, 2003). Anaerobic jars and gas generating kits (Anaerobic system BR 38, Oxoid Ltd., Hampshire, England) were used for creating anaerobic conditions. Plates in duplicate were incubated for 48 h at 37^{o}C for *L. acidophilus, L. casei*, and *Bifidobacterium* spp., 48 h at 42^{o}C for *L. delbrueckii* ssp. *bulgaricus* and aerobically for 48 h at 37^{o}C for *S. thermophilus*. All enumeration techniques followed protocols reported previously (Dave and Shah, 1996; Tharmaraj and Shah, 2003). Plates with 25-250 colonies were counted and recorded as cfu/mL of the fermented milk. The pH changes of batches were monitored before and during fermentation at 0, 3, 6, 9, 12 and 24 h using a pH meter (HANNA Instruments 8417, Singapore).

6.2.5 Organic acids

Organic acid contents were measured according to the procedure described in Section 3.2.5

6.2.6 Proteolytic activity in fermented milk

The degree of proteolysis during fermentation of milk was determined according to the procedure described in Section 3.2.6.

6.2.7 *In vitro* inhibition of angiotensin I-converting enzyme (ACE inhibitory activity)

ACE inhibitory activity was measured according to the procedure described in Section 4.2.6.

6.2.8 Statistical analysis

The experiment was independently replicated three times in a completely randomized design. All results obtained were analysed as a split plot in time design with strains and time as main factors, using the general linear model (GLM) procedure of the SAS System (SAS, 1996). The univariate ANOVA test was validated by fulfilling Huynh-Feldt (H-F) condition (Littell *et al*., 1998). Where appropriate, one-way ANOVA and correlational analyses were employed and the multicomparison of means was assessed by Tukey's test. The statistical level of significance was preset at 0.05 ($P < 0.05$).

6.3 RESULTS AND DISCUSSIONS

6.3.1 Assessment of extracellular and intracellular peptidase activity

Table 6.1 shows the activities of extracellular and intracellular peptidases towards

various peptides detected by TLC. All dairy cultures tested appeared to have produced enzymes capable of hydrolysing large biologically active peptide, bradykinin, at both the extracellular and intracellular levels. The enzymes involved in the hydrolysis may include endopeptidases PepE and PepO and aminopeptidase PepP (Christensen *et al*., 1999). PepE has been identified to hydrolyse the N-terminal and C-terminal blocked substrate N-benzoyl-Phe-Val-Arg-*ρ*NA (Fenster *et al*., 1997). PepE is capable of hydrolysing bradykinin (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg) at the Gly4-Phe5 bond (Christensen *et al*., 1999). Similarly, PepO hydrolysis oligopeptides ranging in length from five to thirty-five including bradykinin (Stepaniak and Fox, 1995). Furthermore, PepP liberates the N-terminal amino acid from peptides with general specificity for Xaa-Pro-Pro-(Yaa)n sequences with high activity reported for peptides ranging from three to nine residues (i.e. bradykinin) but did not hydrolyse dipeptides (Mars and Monnet, 1995). Identified tripeptidase, PepT, has been reported to show no activity for any di-, tetra-, or larger oligopeptides and therefore exhibit strict specificity for tripeptides (Christensen *et al*., 1999). Consequently, the tripeptide substrates Gly-Ala-Tyr and Gly-Leu-Phe tested might have been hydrolysed by PepT. In addition to PepT, aminopeptidases PepC and PepP have also been reported to hydrolyse a variety of tripeptides with uncharged or basic residues in the amino acid terminal position (Christensen *et al*., 1999). However, with the substrate ending with a C-terminal of phenylalanine, Gly-Leu-Phe, the hydrolysis only occurred intracellularly, suggesting the location of these aminopeptidases. This was consistent with the findings of Shihata and Shah (2000) and Tan and Konings (1990). The intracellular aminopeptidases are capable of cleaving N-terminal amino acids from a wide range of peptides differing both in size and composition to release amino acids and peptides (Kunji *et al*., 1996).

Specific tripeptidase and endopeptidase activities of yoghurt culture and selected strains of probiotic organisms assessed towards Gly-Ala-Tyr, Gly-Lue-Phe and oligopeptide bradykinin as substrates is shown in Table 6.2. As opposed to other strains, the hydrolysis of all oligopeptides by *L. acidophilus* La 4962 was significantly ($P < 0.05$) higher at the IE level. This suggested that *L. acidophilus* La 4962 may have produced substantial amounts of PepT and PepE than the other strains which caused the hydrolysis of Gly-Ala-Tyr, Gly-Lue-Phe and oligopeptide bradykinin. Eventhough, all organisms showed specific tripeptidase activity mainly at the IE level, the extent of which was greatly ($P < 0.05$) strain specific confirmed previous reports (Table II) (Kunji *et al*., 1996; Tan and Konings, 1990; Tan *et al*., 1995). The low ($P < 0.05$) EE level of enzyme activity shows that tripeptidase and endopeptidase activities may solely take place intracellularly. The observed extracellular

enzyme activity may be due to cell lysis and release of intracellular enzymes into the medium (Blanc *et al*., 1993; Coolbear *et al*., 1994). The EE values may further indicate variations in the extent of lysis which may be high with some cultures and likely lead to erroneous conclusions (Blanc *et al*., 1993).

X-prolyl-dipeptidyl aminopeptidase, PepX, activity of cell-free extract of individual cultures of bacteria is presented in Figure 6.1. This enzyme is of great importance for the selection of dairy cultures due to high proline content in caseins. All the studied strains exhibited PepX activity towards glycyl-prolyl *p*-nitroanilide (Gly-Pro-pNA) as substrate at both IE and EE cell-free extracts. The IE level of X-prolyl-dipeptidyl aminopeptidase activity was significantly ($P < 0.05$) higher than that at the EE level. This shows that proline-containing peptides were mainly hydrolysed intracellularly by the enzyme (Figure 6.1). Kunji *et al*. (1996) reported the presence of PepX in all species of LAB. Pan *et al*. (2005) similarly identified X-prolyl-dipeptidyl aminopeptidase activity from IE cell-free extract of *L. helveticus* in the hydrolysis of skimmed milk proteins. Fernandez-Espla *et al*. (1997) detected the presence of various proline-specific peptidases in the cell-free extract of *L. casei* ssp. *casei* IFPL 731 and further reported that the enzyme usually cleaves N-terminal X-Pro dipeptides from tri- and oligopeptides. The aminopeptidase activities of microorganisms included in our study may contribute to the production of free amino acids in a fermenting medium as growth factors and peptides, which may have ACE-inhibitory activity (Pan *et al*., 2005).

Aminopeptidase activities of all examined strains towards dipeptide substrates are shown in Table 6.3. The strains studied exhibited on average 71% dipeptidases activity at the IE level showing preference towards N-terminal hydrophobic/uncharged residues resulting in hydrolysis of free amino acids for growth. Leu-Gly, Leu-Tyr, Ala-Met and Ala-His were probably hydrolysed by either one or both aminopeptidases (PepC and PepN) of the studied microorganisms. These enzymes, which have been identified in LAB, were reported to exhibit broad specificity for residues that are basic, acidic, hydrophobic/uncharged and aromatic substrates (Christensen *et al*., 1999). Wohlrab and Bockelmann (1992) also observed that all dipeptidases were active towards numerous dipeptides. PepC and PepN, on the other hand, have been shown to be inactive towards Pro-Xaa dipeptides (Christensen *et al*., 1999). Since PepX has specificity for removal of proline-containing dipeptides (X-Pro ↓ Y…) from the N-termini of peptides (Christensen *et al*., 1999), it may be assumed that PepX was likely not active towards Pro-Ile. Therefore the probable presence of aminopeptidase PepR in the studied strains might have hydrolysed the

tested substrate Pro-Ile from the hydrophobic uncharged C-terminal –Ile. In general, all the organisms showed appreciable aminopeptidase activity at the IE level towards Ala-Met, Leu-Tyr, Leu-Gly, Ala-His, and Pro-Ile, in agreement with the findings of Shihata and Shah (2000). Contrary to our findings, Tan *et al*. (1995) observed very low or no intracellular dipeptidase activity towards Pro-containing dipeptides. Extracellular dipeptidase activity observed in our study was significantly ($P < 0.05$) lower for all substrates than that at the IE level. *Bifidobacterium* spp. showed similarities in their intracellular dipeptidase activities, however, *B. lactis* B94 exhibited higher Pro-Ile and Ala-Met hydrolysis than that of *B. longum* Bl 536 (Table 6.3). This may explain the similarity in their growth pattern (Figure 6.3). Several studies (Kunji *et al*., 1996; Tan and Konings, 1990; Tan *et al*., 1995) have reported dipeptidase activity solely at the IE level. It is interesting to note that there is little information available in the literature on aminopeptidase activities of *Bifidobacterium* spp. Therefore this study is important in this area of enzyme activity and further work in understanding their proteolytic activity and growth in milk is underway.

6.3.2 Proteolytic activity

After analysing segments of proteolytic system in the defined medium, we assessed the proteolytic activities of selected LAB in RSM as presented in Figure 6.2. During fermentation, milk proteins were hydrolysed by LAB proteinases and peptidases resulting in an enhanced amount of free amino groups and peptides (Figure 6.2). Juillard *et al*. (1995b) reported that the level of free amino acids and peptides in milk is low therefore LAB depend on a proteolytic system that allows for an efficient degradation of milk proteins. The presence of aminopeptidases is important for the release of amino acids for growth by microorganisms through the hydrolysis of peptides in the growth medium. Christensen and Steele (2003) demonstrated that the loss of selected aminopeptidase (PepC, PepN, and PepX) activities resulted in significant impairment of growth rate in milk.

The extent of proteolysis varied among strains and appeared to be time dependant. As depicted in Figure 6.2, the amount of liberated amino groups and peptides increased only slightly during fermentation from 0 to 12 h for some strains (*L. acidophilus* L 10, *L. acidophilus* La 4962, *B. lactis* B94, *B. longum* Bl 536, *L. casei* L 26, and *L. casei* Lc 279) but increased significantly ($P < 0.05$) for all strains from 12 to 24 h (Figure 6.2). These findings were consistent with those reported by Nielsen *et al*. (2001). In contrast, however, Leclerc *et al*. (2002) reported a linear increase in the amount of free amino groups until the end of fermentation in milk fermented with *L. helveticus* strains. The proteolytic pattern

certainly had a strong effect on bacterial growth due to correlation between these parameters, which ranged from 0.76 for L26 to 0.90 for St 1342. This dependence could possibly explain the general slow cell growth up to 6 h of fermentation at 42^{o}C. Although *L. delbrueckii* ssp. *bulgaricus* Lb 1466 showed appreciable peptidase activity, it experienced poor growth. This indicated that this organism might require some other growth factors in addition to free amino acids and peptides. *Lactobacillus casei* L26 showed the highest proteolytic activity followed by *L. delbrueckii* ssp. *bulgaricus* Lb 1466, *S. thermophilus* St 1342 and *L. acidophilus* (La 4962 and L10), *Bifidobacterium* and *L. casei* (Lc279) (Figure 6.2) with the activity apparently strain specific ($P<0.0001$). Similar to our findings, those of Shihata and Shah (2000), Fuglsang *et al*. (2003) also showed that the amount of free amino groups formed in the medium during fermentation was strain dependent. Thus, the differences in the amounts of amino groups released during fermentation of milk observed for the microorganisms, could probably relate to the different proteinases of the strains. Shihata and Shah (2000) reported proteolytic activity of *S. thermophilus*, *L. delbrueckii* ssp. *bulgaricus*, and *L. acidophilus* to be much greater than that of *Bifidobacterium* spp. The results in our study also showed similar pattern of proteolytic activity for *S. thermophilus* St 1342, *L. delbrueckii* ssp. *bulgaricus* Lb 1466, *L. acidophilus* strains (La 4962 and L10) and *L. casei* L26 being higher than that of *Bifidobacterium* spp. at 24 h fermentation.

6.3.3 Cell growth and organic acids production

The change of cell concentration of selected dairy LAB and probiotic organisms cultivated individually in RSM for 24 h at 42^{o}C is shown in Figure 6.3. Lactic acid bacteria are nutritionally fastidious organisms which require more free amino acids or peptides than present in milk. Thus proteolytic activity is important requirement for achieving a minimum level of 10^6 to 10^7 cfu/mL of active probiotics in a product to observe positive health effect (Shah, 2000b; Talwalkar and Kailasapathy, 2004). In general, examined probiotic cultures achieved the desired therapeutic level (10^8 cfu/mL) during their growth in RSM for 24 h (Figure 6.3). Although the cultures showed a consistent increase in cell concentration until 9 h, the required pH of 4.5 was not reached as shown in Figure 6.4. Sodini *et al*. (2002) also reported that individual probiotic cultures grew well in fermented milk and did not produce organic acids as fast as with mixed starter cultures. *Lactobacillus acidophilus* 4962 and *L. casei* Lc 279 experienced a superior growth ($P < 0.05$) as compared to other probiotic organisms. On the other hand, *B. lactis* B94 and *B. longum* Bl 536 showed slow growth pattern and no significant ($P > 0.05$) difference in the cell counts was observed (Figure 6.3).

S. thermophilus St 1342 achieved a significantly ($P < 0.05$) higher cell concentration in comparison to *L. delbrueckii* ssp. *bulgaricus* Lb1466; the slow growth of the latter resulted in low viability (10^7 cfu/mL) at the end of the fermentation period of 20 h.

Eventhough *L. delbrueckii* ssp. *bulgaricus* Lb 1466 grew slowly in RSM, it produced substantial amount of lactic acid, second to *S. thermophilus* St1342 (Figure 6.5), and the highest ($P < 0.05$) decline in pH at the end of fermentation (Figure 6.4). As previously observed in Section 3.3.3, the yoghurt culture (*S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus*) produced substantially more lactic acid than any other strain in the study. However, the study also showed that probiotic organisms produced some lactic acid even though not as high as in the case of yoghurt culture, with *L. acidophilus* L10 producing the highest and *L. casei* L26 the lowest concentration (Figure 6.5).

In general, the production of acetic acid as presented in Figure 6.6 was fairly uniform in each batch of fermented milk up to 12 h. All cultures produced a substantial ($P < 0.05$) amount of acetic acid except *L. delbrueckii* ssp. *bulgaricus* Lb 1466 (Figure 6.6). *Lactobacillus casei* Lc 279 on the other hand, produced significantly ($P < 0.05$) higher acetic acid than other probiotics. The upsurge in cell growth for all batches from 9 h to 24 h (Figure 6.3) resulted in significant ($P < 0.05$) increases in the concentration of organic acids and decline in pH (Figure 6.4) but the concentration of organic acids attained did not affect the cell growth as noted in other studies (Akalin *et al*., 2004; Shah, 2000b; Sodini *et al*., (2002). The organisms appeared to have maintained appreciable cell counts during the 24 h fermentation in RSM possibly due to efficient proteolytic systems (Juillard *et al*., 1995b).

6.3.4 ACE-inhibitory activity

In our study, eight strains of LAB were cultured individually in RSM to produce fermented milk with *in vitro* ACE-inhibitory activity shown in Table 6.4. The results indicate that the production of ACE inhibitors was not confined to a single species or strains of bacteria but all the strains tested produced peptides, which showed *in vitro* ACE-inhibitory activity. However, un-inoculated RSM did not show ACE-I activity (data not shown). Furthermore, these selected microorganisms showed X-prolyl-dipeptidyl aminopeptidase activity, which was able to cleave proline-containing sequences (Figure 6.1). This may mean that specific peptides could be produced by cleaving N-terminal of X-Pro dipeptides from tri- and oligopeptides which may show ACE-I activity (Kunji *et al*., 1996). The ACE-I activity of the fermented milk obtained for the selected LAB was also compared by determining the protein concentration needed to inhibit 50% of the original

ACE activity (IC_{50}) (Table 6.4). There was a significant ($P < 0.05$) difference in IC_{50} values between the bacterial strains, indicating possible differences in the quality of ACE-inhibitory peptides produced by the organisms. *B. longum* Bl 536 and *L. acidophilus* L10 produced the highest activity at 63.7% and 62.6% with IC_{50} values of 0.196 and 0.151 mg/mL, respectively. Similar results were reported by Pihlanto-Leppalla *et al*. (1998) using different lactic acid staters and digestive enzymes to obtain inhibition of 35 to 86% and IC_{50} values between 0.080 to 0.314 mg/mL. Several milk protein-derived peptides produced by the enzymatic hydrolysis (Roy *et al*., 2000) or by the fermentation of milk with different strains of organisms (Gobbetti *et al*., 2002; Leclerc *et al*., 2002) inhibited the activity of ACE.

Although the proteolytic activity of *B. longum* Bl 536 was not as high as *L. casei* L26, the peptides released by *B. longum* Bl 536 had higher ACE-inhibitory activity than those of *L. casei* L26 (Table 6.4). Fuglsang *et al*. (2003) found that most LAB produced ACE inhibitors in varying amounts during milk fermentation which varied with strains. Their study was in line with our findings which showed a similar trend using selected strains of bacteria and further reported that only *L. helveticus* produced such substances in amounts large enough to cause a direct effect on ACE *in vivo* out of several strains used. The time-dependent release of various peptides observed in our study might have important consequences on the extent of *in vitro* ACE inhibitory activity in fermented milk, which deserves further elaboration.

6.4 CONCLUSION

Selected cultures of *S. thermophilus* St1342, *L. delbrueckii* ssp. *bulgaricus* Lb 1466, *L. acidophilus* L10, *B. lactis* B94, *L. casei* L26, *L. acidophilus* La 4962, *B. longum* Bl 536 and *L. casei* Lc 279 were capable of growing in milk likely due to degradation of milk proteins. Thus, this study has shown that for active and rapid growth in milk, dairy cultures may rely on their proteolytic system. The cultures possessed proteolytic enzymes – protinases, peptidases and aminopeptidases at extracellular and intracellular levels which showed specific activities toward certain substrates. All strains exhibited X-prolyl-dipeptidyl aminopeptidase activity cleaving proline-containing sequences. The proteolytic systems including X-prolyl-dipeptidyl aminopeptidase activity of the bacteria strains in RSM resulted in the release of free amino groups and peptides which substantially improved growth and maintained viability in the medium. The proteolytic activity did not appear as the governing factor of growth for *Lactobacillus delbrueckii* ssp. *bulgaricus,* which might

require additional growth factors. All selected strains produced a range of bioactive peptides with varying degree of ACE-inhibition. *B. longum* Bl 536 and *L. acidophilus* L10 produced the highest activity IC_{50} values of 0.196 and 0.151 mg/mL, respectively. ACE-I was strain and apparently time dependent.

Table 6.1 Aminopeptidase activities of single cultures (*S. thermophilus* St1342, *L. delbrueckii* ssp. *bulgaricus* Lb 1466, *L. acidophilus* (L10 and La 4962), *B. lactis* B94, *B. longum* Bl 536, *L. casei* (L26 and Lc 279)

	Aminopeptidase activity	
Substrates	EE[α]	IE[β]
Tripeptides		
Gly-Ala-Tyr	+[#]	+
Gly-Leu-Phe	-[*]	+
Oligopeptide		
Bradykinin	+	+

Hydrolysis of peptides was analysed by TLC; [#] + = hydrolysis; -* = no hydrolysis; [α]EE = cell wall extract; [β]IE = intracellular extract.

Table 6.2 Tripeptidase specific activity of yoghurt culture and selected strains of probiotic organisms after 20 h of single culture fermentation in MRS at 42°C

Culture	Tripeptidase specific activity, U/mg protein		
	Substrates		
	Gly-Ala-Tyr	Bradykinin	Gly-Leu-Phe
S. thermophilus			
St1342, EE	370.79 ± 1.14 ^{a}	349.32 ± 0.38 ^{b}	359.97 ± 0.72 ^{c}
IE	555.09 ± 0.40 ^{a}	534.32 ± 0.70 ^{b}	551.30 ± 0.91 ^{c}
L. delbrueckii ssp. *bulgaricus* Lb1466			
EE	441.06 ± 6.91 ^{a}	500.88 ± 0.96^{b}	449.36 ± 0.46 ^{a}
IE	770.45 ± 0.42 ^{a}	671.99 ± 1.57 ^{b}	700.76 ± 1.52 ^{c}
L. acidophilus L10			
EE	218.20 ± 2.30 ^{Aa}	246.80 ± 0.51 ^{Ab}	252.11 ± 0.51 ^{Ab}
IE	557.47 ± 0.46 ^{Aa}	521.10 ± 1.18 ^{Ab}	485.40 ± 1.66 ^{Ac}
L. acidophilus La 4962			
EE	704.52 ±32.73 ^{aB}	611.14 ± 7.76 ^{Bab}	675.21 ± 0.29 ^{Bc}
IE	2844.43 ± 3.45 ^{aB}	2921.26 ± 2.84 ^{Bb}	3021.52 ± 1.13 ^{Bc}
B. lactis B94			
EE	671.96 ± 3.63^{Aa}	180.72 ± 1.46 ^{Ab}	209.78 ± 2.81 ^{Ac}
IE	1516.73 ± 9.44 ^{Aa}	1360.36 ± 3.12 ^{Ab}	1263.60 ± 1.80 ^{Ac}
B. longum Bl536			
EE	628.39 ± 2.57 ^{Ba}	656.43 ± 2.08 ^{Bb}	583.77 ± 2.19 ^{Bc}
IE	913.84 ± 0.96 ^{Ba}	741.68 ± 0.34 ^{Bb}	724.69 ± 0.91 ^{Bc}
L. casei L26			
EE	289.78 ± 2.74 ^{Aa}	414.14 ± 4.20 ^{Ab}	726.36 ±2.11^{Ac}
IE	899.92 ± 1.05 ^{Aa}	907.47± 9.60 ^{Aab}	927.82 ± 0.23 ^{Ab}
L. casei Lc279			
EE	470.76 ± 29.51 ^{Ba}	573.84 ± 23.27 ^{Ba}	467.94 ± 49.42 ^{Ba}
IE	646.27 ± 0.80 ^{Ba}	644.55± 0.32 ^{Ba}	652.03 ± 0.64 ^{Bb}

Results presented as a mean of three observations. Significant when $P < 0.05$; Tripeptidase activity expressed as specific activity defined as units of enzyme activity per milligram of protein in crude cellular extract. EE = extracellular cell wall extract; IE = intracellular extract; ^{abc} Means in the same row with different small letter superscripts are significantly different; ^{AB} Means in the same column for particular strains with different capital letter superscripts are significantly different.

Table 6.3 Dipeptidase specific activities of single strains of selected probiotic organisms and yoghurt culture after 20 h of individual fermentations in MRS at 42°C

Culture	Dipeptidase specific activity, U/mg protein				
	Substrates				
	Leu-Gly	Leu-Tyr	Pro-Ile	Ala-Met	Ala-His
S. thermophilus St1342					
EE	90.69 ± 10.78^{a}	82.20 ± 22.34^{a}	71.58 ± 0.30^{a}	92.81 ± 14.56^{a}	93.72 ± 17.36^{a}
IE	271.35 ± 0.99^{a}	329.20 ± 4.36^{b}	317.26 ± 0.64^{b}	283.51 ± 2.01^{a}	283.05 ± 1.89^{a}
L. delbrueckii ssp. *bulgaricus* Lb 1466					
EE	101.94 ±15.08^{a}	84.68 ± 4.17^{a}	89.75 ± 30.13^{a}	72.76 ± 33.07^{a}	76.63 ± 27.36^{a}
IE	265.66 ± 4.62^{a}	282.77 ± 1.31^{ab}	281.17 ± 2.48^{a}	239.77 ± 2.99^{bc}	335.36 ± 3.53^{bcd}
L. acidophilus L10					
EE	81.36 ± 5.52^{Aa}	105.17 ± 16.29^{Aa}	131.99 ± 1.38^{Aa}	86.79 ± 20.34^{Aa}	114.81 ± 15.37^{Aa}
IE	323.73 ± 2.99^{Aa}	234.27 ± 2.99^{Ab}	247.54 ± 5.39^{Ab}	318.95 ± 2.04^{Aa}	292.01 ± 1.27^{Abc}
L. acidophilus La 4962					
EE	241.78 ± 35.50^{Ba}	297.55 ± 5.04^{Ba}	270.50 ± 43.51^{Ba}	277.85 ±25.38^{Ba}	207.38 ± 28.73^{Ba}
IE	1127.62 ± 4.69^{Ba}	1097.02 ± 2.84^{Ba}	1028.01 ± 6.41^{Bb}	1081.39 ± 17.91^{Ba}	917.33 ± 5.33^{Bbc}
B. lactis B94					
EE	114.99 ± 17.53^{Aa}	56.25 ± 4.23^{Aa}	122.18 ± 27.87^{Aa}	107.80 ± 32.97^{Aa}	86.56 ± 18.95^{Aa}
IE	407.80 ± 4.13^{Aa}	469.81 ± 4.01^{Ab}	578.82 ± 6.89^{Abc}	518.18 ± 3.59^{Abcd}	385.31 ± 2.36^{Aa}
B. longum Bl 536					
EE	103.41 ± 32.51^{Aa}	60.19 ± 20.80^{Aa}	129.68 ± 28.28^{Aa}	129.35 ± 25.96^{Aa}	66.17 ± 22.87^{Aa}
IE	340.20 ± 4.47^{Ba}	362.34 ± 5.40^{Ba}	320.63 ± 2.89^{Bab}	355.82 ± 5.08 ^{Ba}	311.36 ± 12.19^{Bab}
L. casei L26					
EE	124.17 ± 3.53^{Aa}	64.73 ± 6.38^{Aa}	87.76 ± 15.02^{Aa}	101.14 ± 5.18^{Aa}	67.53 ± 30.21^{Aa}
IE	417.04 ± 14.67^{Aa}	277.57 ± 1.87^{Ab}	280.31 ± 0.82^{Ab}	307.98 ± 1.19^{Ab}	313.01 ± 8.77^{Ab}
L. casei Lc 279					
EE	63.50 ± 33.45^{Ba}	106.71 ± 18.19^{Ba}	92.63 ± 19.45^{Aa}	91.32 ± 2.47^{Aa}	78.23 ± 0.87^{Aa}
IE	243.10 ± 0.61^{Ba}	290.32 ± 1.74^{Ab}	292.04 ± 0.98^{Ab}	288.61 ± 2.45^{Ab}	286.15 ± 2.33^{Ab}

Results presented as a mean of three observations. Significant when $P < 0.05$; Dipeptidase activity expressed as specific activity which is defined as units of enzyme activity per milligram of protein in crude cellular extract. EE = extracellular cell wall extract; IE = intracellular extract. ^{abcd} Means in the same row with different small letter superscripts are significantly different; ^{AB} Means in the same column for particular strains with different capital letter superscripts are significantly different.

Table 6.4 Angiotensin I-converting enzyme (ACE) inhibitory of fermented milk extracts obtained after 24 h of individual culture fermentations in RSM at 42°C

In vitro ACE-inhibitor activity		
Culture	24 h Inhibition, %	IC_{50}*, mg/mL
S.thermophilus St1342	43.65 ± 4.67	0.225± 0.033
L. delbrueckii ssp. *bulgaricus* Lb1466	51.10 ± 2.30	0.187± 0.012
L. acidophilus L10	62.55 ± 0.24 ^{A}	0.151± 0.001^{A}
L.acidophilus La4962	53.58 ± 1.73 ^{B}	0.164± 0.007^{B}
B. lactis B94	51.64 ± 3.63 ^{A}	0.175± 0.016^{A}
B. longum Bl536	63.73 ± 1.71 ^{B}	0.196± 0.007^{A}
L. paracasei L26	54.02 ± 1.71 ^{A}	0.196± 0.008^{A}
L. casei Lc279	41.15 ± 4.13 ^{B}	0.344± 0.053^{B}

*The activity is expressed as the concentration of peptides in mg/mL required to inhibit 50% of the original ACE activity (IC_{50}). AB Means in the same column for particular strains with different capital letter superscripts are significantly different.

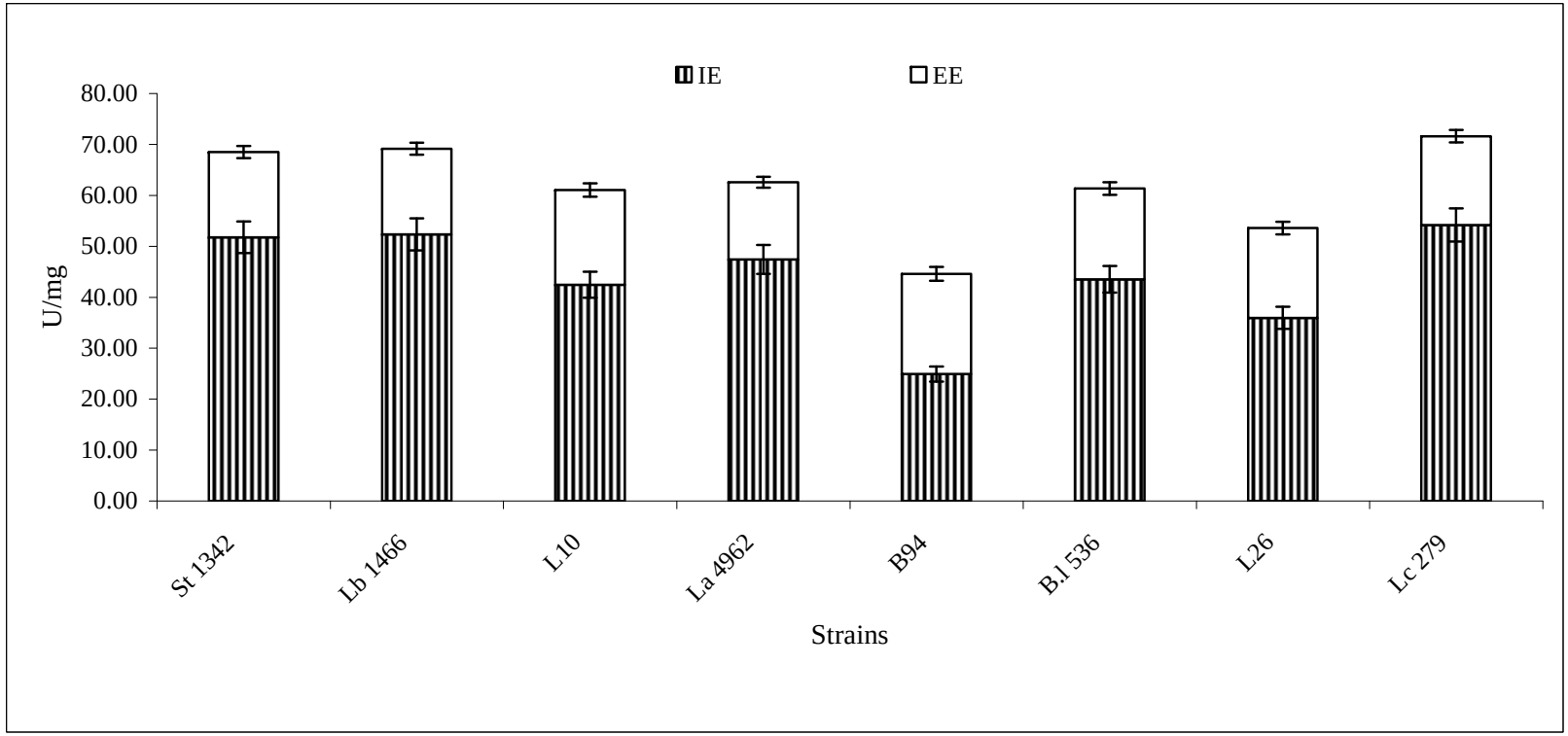

Figure 6.1 X-prolyl-dipeptidyl aminopeptidase activity of cell-free intracellular (IE) and extracellular (EE) enzymatic extracts of individual cultures of bacteria (*S. thermophilus* St1342, *L. delbrueckii* ssp. *bulgaricus* Lb 1466, *L. acidophilus* L10, *L. acidophilus* La 4962, *B. lactis* B94, *B. longum* Bl 536, *L. casei* L26 and *L. casei* Lc 279) using Gly-Pro-pNA as the substrate in 50 mM Tris-HCl buffer pH 7.0 at 37^{o}C. Aminopeptidase specific activity is defined as units (U) of enzyme activity per mg of protein in crude cellular extract. One unit of enzyme activity was defined as the amount of enzyme required to release 1 µmol of *p*-nitroanilide per min under assay conditions. (Error bars represent a pooled standard error of the mean).

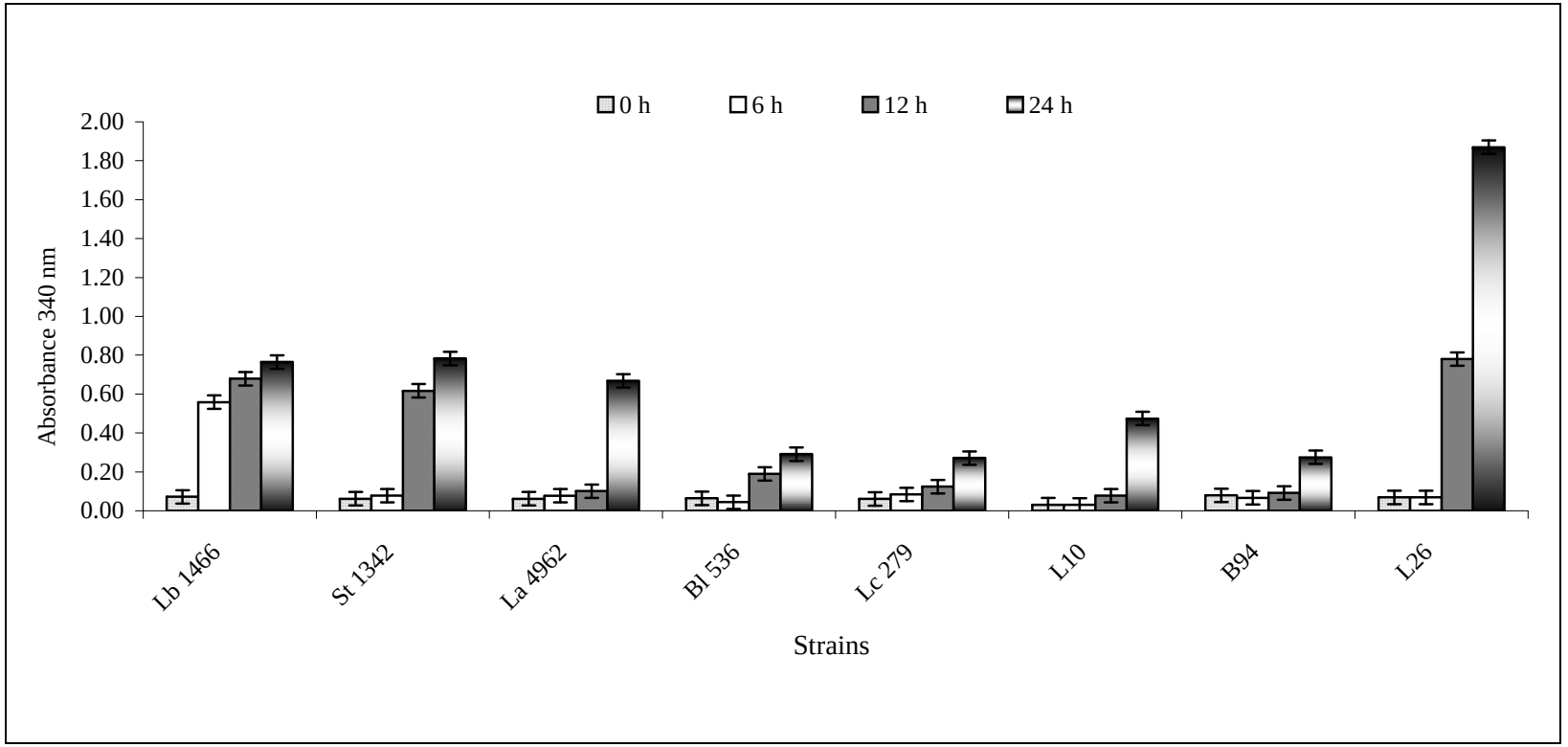

Figure 6.2 Proteolytic activity by single strains of bacteria (*L. delbrueckii* ssp. *bulgaricus* Lb 1466, *S. thermophilus* St1342, *L. acidophilus* La 4962, *B. longum* Bl 536, *L. casei* Lc 279, *L. acidophilus* L10, *B. lactis* B94 and *L. casei* L26) during 24 h fermentation in RSM at 42^{o}C (Error bars represent a pooled standard error of the mean SEM = 0.03 absorbance units).

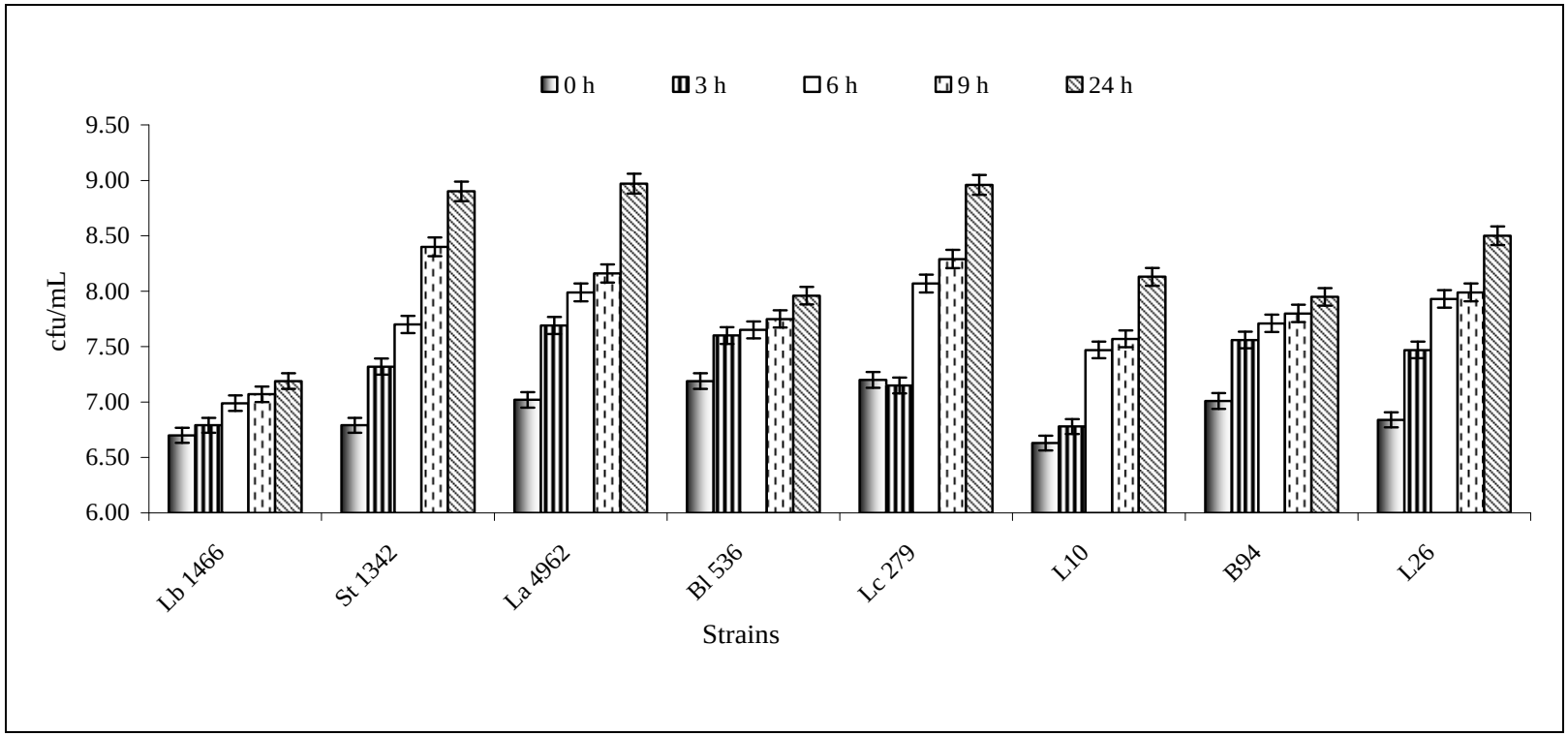

Figure 6.3 The change of cell concentration of selected dairy LAB and probiotic organisms (*L. delbrueckii* ssp. *bulgaricus* Lb 1466, *S. thermophilus* St1342, *L. acidophilus* La 4962, *B. longum* Bl 536, *L. casei* Lc 279, *L. acidophilus* L10, *B. lactis* B94 and *L. casei* L26) cultivated in reconstituted skim milk (RSM) for 24 h at 42^{o}C (Error bars represent a pooled standard error of the mean, SEM = 0.11 cfu/mL).

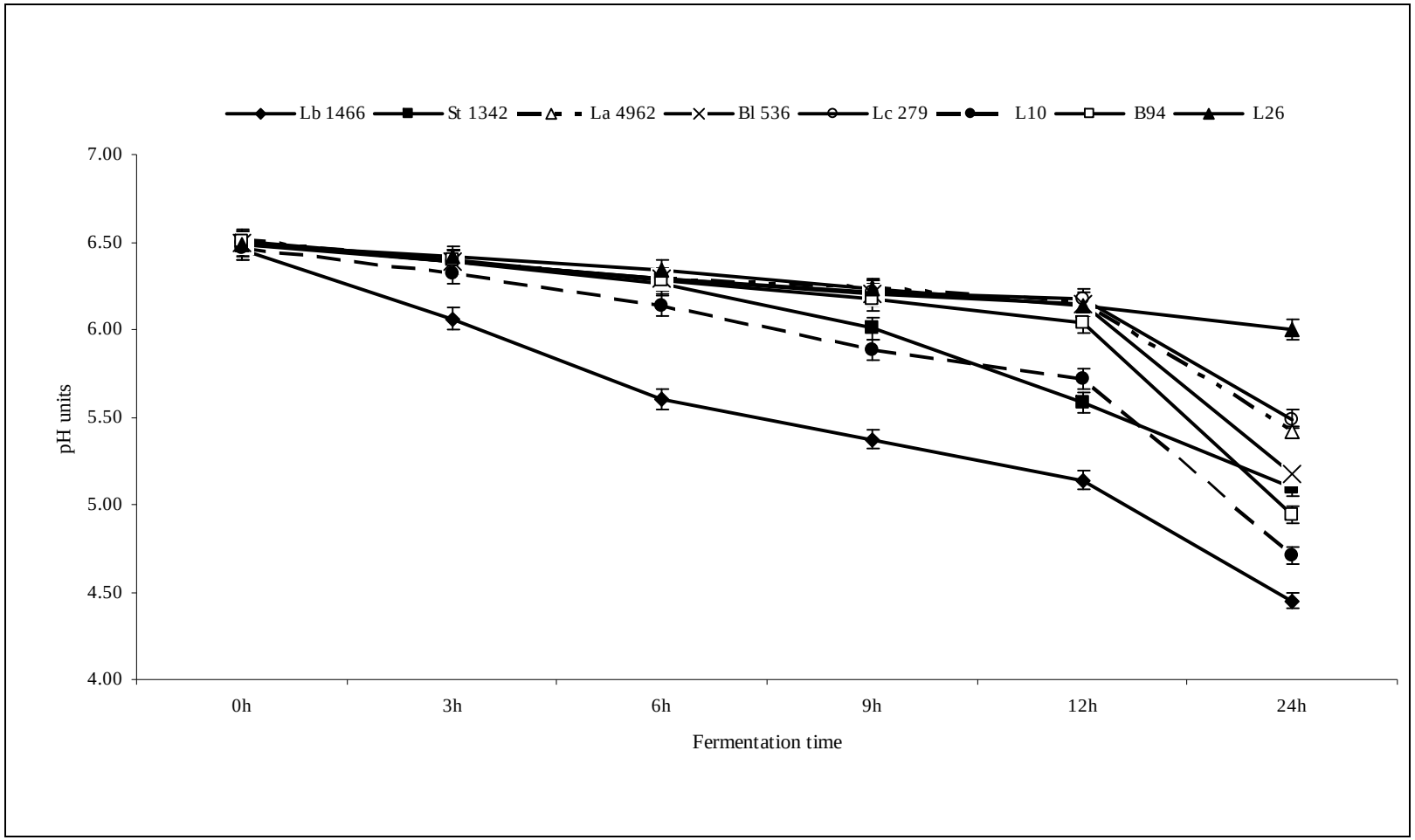

Figure 6.4 pH changes during fermentation of single strains in RSM by *L. delbrueckii* ssp. *bulgaricus* Lb 1466, *S. thermophilus* St1342, *L. acidophilus* La 4962, B.longum Bl 536, *L. casei* Lc 279, *L. acidophilus* L10, *B. lactis* B94 and *L. casei* L26. (Error bars present a pooled standard error of the mean).

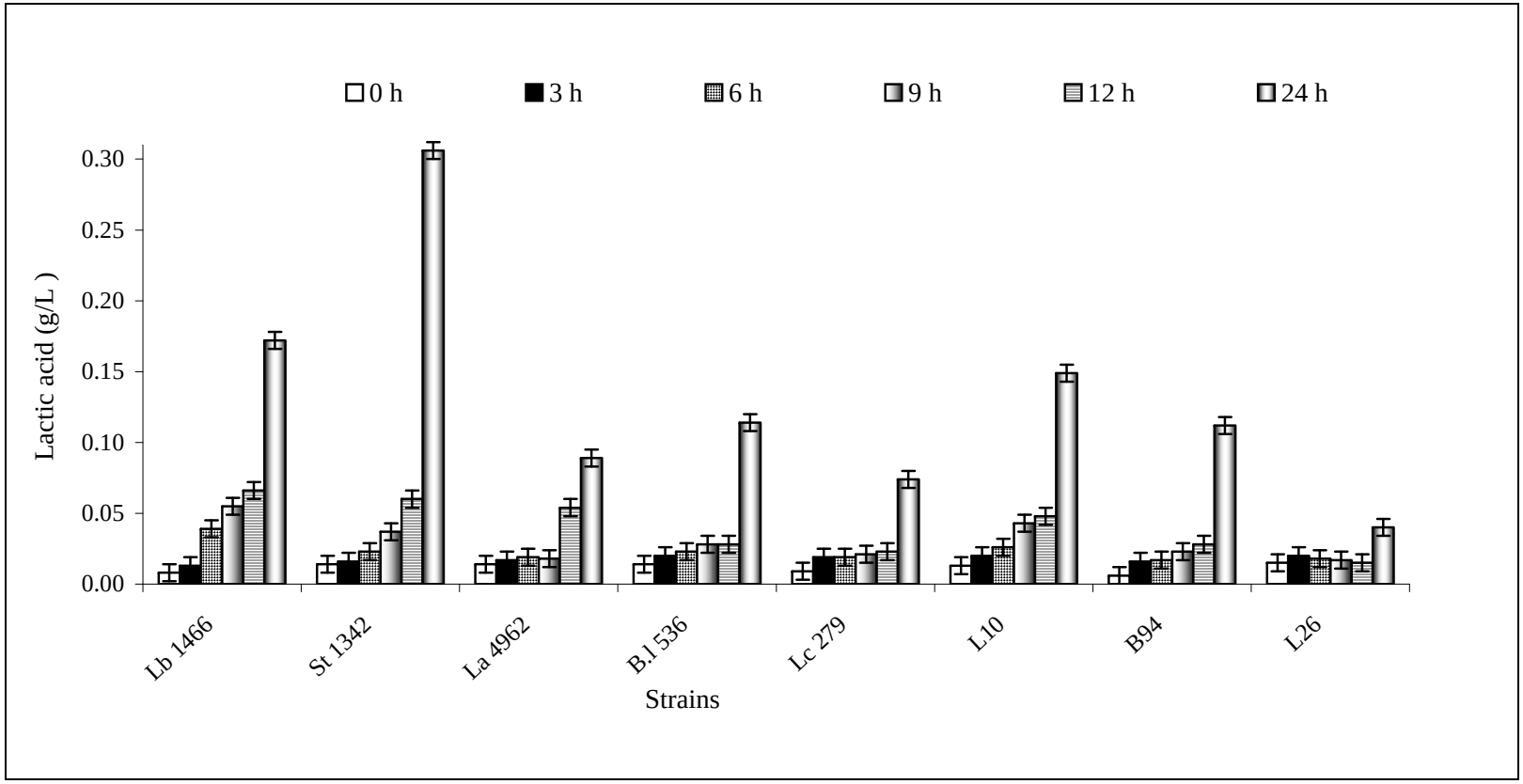

Figure 6.5 Production of lactic acid during fermentation of single strains in RSM for 24 h at 42^{o}C by *L. delbrueckii* ssp. *bulgaricus* Lb 1466, *S. thermophilus* St1342, *L. acidophilus* La 4962, *B. longum* Bl 536, *L. casei* Lc 279, *L. acidophilus* L10, *B. lactis* B94 and *L. casei* L26. (Error bars represent a pooled standard error of the mean SEM = 0.01 g/L).

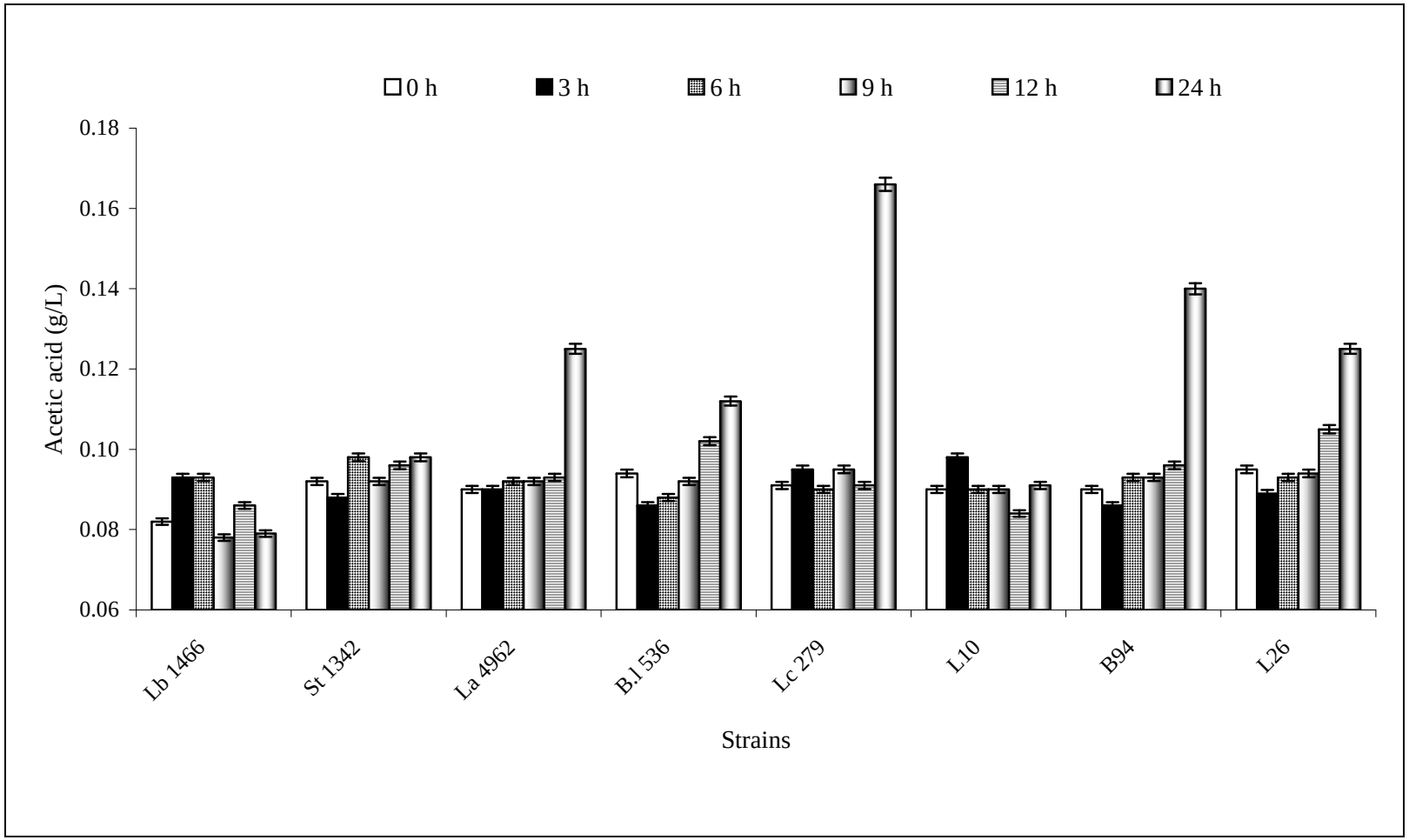

Figure 6.6 Production of acetic acid in fermented milk by *L. delbrueckii* ssp. *bulgaricus* Lb 1466, *S. thermophilus* St1342, *L. acidophilus* La 4962, *B. longum* Bl 536, *L. casei* Lc 279, *L. acidophilus* L10, *B. lactis* B94 and *L. casei* L26 during 24 h fermentation of RSM at 42^{o}C (Error bars represent a pooled standard error of the mean SEM = 0.01 g/L).

7.0 α-Galactosidase and proteolytic activities of selected probiotic and dairy cultures in fermented soymilk

INTRODUCTION

Soy-based foods may provide a range of health benefits to consumers due to their hypolipidemic, anticholesterolemic and antiatherogenic properties as well as due to reduced allergenicity (Favaro Trindade *et al*., 2001). It also contains isoflavones, which have been linked to reduced risk of most hormone-associated health disorders (Kurzer, 2000). However, consumption of soymilk is hindered due to the presence of unpleasant off-flavors carried over from soy beans. These characteristic flavors are caused by *n*-hexanal and pentanal, which occur in beans as a product of breakdown of unsaturated fatty acids (Arai *et al*., 1996; Scalabrini *et al*., 1998). In addition to these aldehydes, soymilk contains various oligosaccharides including raffinose and stachyose that may cause a gastrointestinal discomfort to consumers (Scalabrini *et al*., 1998; Shin *et al*., 2000).

Raffinose and stachyose are α-galactosides of sucrose comprising three and four monomeric units respectively and are non-digestible in the gut due to the absence of α-galactosidase in the human intestinal mucosa. Consequently, intact oligosaccharides pass directly into the lower intestine where they are metabolized by bacteria that possess this enzyme, resulting in the production of gases (Tsangalis and Shah, 2004). This problem could be alleviated by using a specific enzyme, α-galactosidase or an organism that possesses high α-galactosidase activity to minimize the content of flatulence-causing oligosaccharides in the product (Scalabrini *et al*., 1998). Several *Bifidobacterium* strains have been reported to produce varying levels of α-galactosidase, which metabolized α-galactosyl oligosaccharides in soymilk (Scalabrini *et al*., 1998). Soymilk is a good medium for growing *Bifidobacterium* because it contains oligosaccharides that are fermented by most of the strains belonging to this genus (Liu, 1997; Scalabrini *et al*., 1998).

Bifidobacterium sp., *Lactobacillus acidophilus*, and *Lactobacillus casei* have been associated with health-promoting effects and are classified as probiotic organisms since they are thought to improve the microbial balance in the human gastrointestinal tract (GIT) (Schrezenmeir and De Vrese, 2001). Health benefits attributed to probiotics include antimicrobial, antimutagenic, anticarcinogenic and antihypertensive properties (Lourens-Hattingh and Viljoen, 2001). Antihypertension has been reported to be mediated through inhibition of angiotensin converting enzyme (ACE) (Nakamura, *et al*., 1995). This enzyme

Donkor, O. N., Henriksson, A., Vasiljevic, T., & Shah, N. P. (2007). α-Galactosidase and proteolytic activities of selected probiotic and dairy cultures in fermented soymilk. *Food Chemistry*, *104*, 10-20.

plays a major role in the regulation of blood pressure. ACE converts angiotensin-I to a vasoconstrictor angiotensin-II, and the inactivation of the vasodilator bradykinin. ACE inhibition results in an antihypertensive effect (Saito *et al*., 2000; Fuglsang *et al*., 2003). Many ACE inhibitory peptides have been derived from food proteins (Saito *et al*., 2000; Wu and Ding 2001).

The ability of lactic acid bacteria (LAB) to ferment the available carbohydrates in a growing medium varies with strains. Matsuoka *et al*. (1968) found that *S. thermophilus* produced a greater amount of acid in soymilk than *Lactococcus lactis* and *L. delbrueckii* ssp. *bulgaricus.* Mital *et al*. (1974) also reported that certain organisms such as *S. thermophilus*, *L. acidophilus, L. cellobiosis* and *L. plantarum* which utilize sucrose, exhibited significant growth and produced substantial amounts of acid in soymilk. Others such as *L. delbrueckii* ssp. *bulgaricus* grew poorly in soymilk because of their inability to ferment sucrose and other carbohydrates in soymilk. A similar finding was also reported by Wang *et al*. (1974). The use of LAB in preparing fermented soy products has received much attention (Lee *et al*., 1990; Cheng *et al*., 1990; Karleskind *et al*., 1991; Sherkat *et al*., 2001). Several studies on α-galactosidase activity and metabolism of α-galactosyl oligosaccharides by *Bifidobacterium* strains in soymilk have been reported but there is a lack of detailed information in the literature about the behavior of probiotic organisms (*L. acidophilus* and *L. casei*) and their importance as a part of the starter cultures for making fermented soy products.

The aims of this study were (a) to assess the suitability of soymilk as a substrate for growth and acid development by selected probiotic organisms and by *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus*, (b) to examine the metabolism of oligosaccharides by these selected organisms, and (c) to monitor their proteolytic and ACE-inhibitory activities in soymilk.

7.2 MATERIALS AND METHODS

7.2.1 Bacterial cultures

Pure strains of *L. acidophilus* LAFTI® L10, *B. lactis* LAFTI® B94 and *L. casei* LAFTI® L26 were kindly provided by DSM Food Specialties (Moorebank, NSW, Australia). *S. thermophilus* St1342, *L. delbrueckii* ssp. *bulgaricus* Lb 1466, *L. acidophilus* La 4962, *B. longum* Bl 536 and *L. casei* Lc 279 were obtained from the culture collection of Victoria University (Werribee, Australia). The lyophilized organisms were propagated in deMann Rogosa Sharpe (MRS) broth (Oxoid, West Heidelberg, Australia) according to the

manufacturer's instructions at 37°C with the exception of *L. delbrueckii* ssp. *bulgaricus* Lb 1466 which was grown at 42°C. For propagation of *Bifidobacterium*, sterile MRS broth was supplemented with 0.05% (w/v) L-cysteine.hydrochloride to provide anaerobic condition and to stimulate their growth (Ravula and Shah, 1998). After three successive transfers in MRS, the activated organisms were inoculated at 1% (v/v) level into 10 mL sterilized commercial soymilk (Simply Soy, Sanitarium, NSW, Australia) supplemented with 2% (w/v) glucose and 1% (w/v) yeast extract for the manufacturing of the fermented soymilk.

7.2.2 Extraction of crude α-galactosidase

One of the requirements for good growth of cultures in soy-based media is the activity of α-galactosidase. All organisms (*L. acidophilus* L10, *B. lactis* B94, *L. casei* L26, *S. thermophilus* St1342, *L. delbrueckii* ssp. *bulgaricus* Lb 1466, *L. acidophilus* La 4962, *B. longum* Bl 536 and *L. casei* Lc 279) were assessed for α-galactosidase activity according to the methods of Scalabrini *et al*. (1998) and Tsangalis and Shah (2004). Briefly, the organisms were activated by two successive propagations in MRS broth at 37°C for 20 h. Subsequently, 5% (v/v) of active culture was inoculated into 250 mL of MRS broth and incubated at 37°C for 48 h. In order to ascertain the metabolic characteristics of probiotic and *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus* in soymilk, α–galactosidase activity was examined in MRS basal broth supplemented with 2% (w/v) glucose, 2% (w/v) raffinose or a mixture of 1% (w/v) each of raffinose and glucose. During fermentation, 50 mL aliquots were withdrawn aseptically at 6, 12, 24, and 48 h and stored at 2°C. Bacterial cells were harvested by centrifuging at 4000 x g for 10 min at 4°C using a Sorvall RT7 refrigerated centrifuge (Newtown, Conn., USA). The cell pellet was washed in 20 mL cold 50 mM sodium citrate buffer (pH 5.5) and centrifuged at 4000 x g for 10 min and this was repeated twice. Finally cells were resuspended in 10 mL of the same buffer, placed in an ice bath and sonicated (Unisonics, Pty Ltd. Sydney, Australia) three times for 5 min. The cell debris was removed by centrifugation at 10,000 x g for 30 min at 4°C. The supernatant was used as a crude enzyme extract.

7.2.3 Assay for α-galactosidase

Crude enzyme extracts from the organisms were assayed for α-galactosidase activity according to the method of Scalabrini *et al*. (1998). Briefly, 250 µL of crude enzyme extract was mixed with 500 µL of 5 mM p-nitrophenyl-α-D-galactopyranoside (*p*NPG) and incubated at 37°C for 30 min. The reaction was stopped by addition of 500 µL of cold 0.2 M

sodium carbonate. The α-galactosidase activity was determined by the rate of hydrolysis of *p*NPG. The amount of p-nitrophenol released was measured with a spectrophotometer (LKB NOVASPEC II, Pharmacia, LKB Biochrom, England) at 420 nm. One unit of enzyme activity was defined as the amount of enzyme that released 1 µmol of *p*-nitrophenol from *p*NPG per millilitre per min under assay conditions. The specific activity was expressed as units (U) of α-galactosidase activity per milligram of protein.

7.2.4 Determination of protein concentration

The protein concentration of the crude enzyme extracts was estimated as described in Section 6.2.2.2.

7.2.5 Organic acids production

Analysis of organic acids was performed according to the procedure described in Section 3.2.5.

7.2.6 Performance of dairy cultures in soymilk

7.2.6.1 Fermentation of soymilk

Eight batches of 250 mL of commercial soymilk were heat treated (100^{o}C for 30 min) and aseptically inoculated with 1% (v/v) of each culture and incubated at 42^{o}C for 48 h. All fermentations were performed in triplicate. A control consisted of uninoculated soymilk. During fermentation, aliquots from each batch were taken at 0, 6, 12, 24, and 48 h to monitor cell growth, pH changes, organic acids production, metabolism of oligosaccharides, and proteolytic and ACE-inhibitory activities.

7.2.6.2 Cell Growth

The cell growth of each organism was assessed by enumerating bacterial population during 48 h of fermentation in soymilk as described in the procedure in Section 3.2.4.

7.2.6.3 pH changes and production of organic acids

During culture growth, the main metabolic products are organic acids, particularly lactic and acetic acids. The pH changes in batches of soymilk were monitored during fermentation at 0, 6, 12, 24 and 48 h using a pH meter (HANNA instruments 8417, Singapore). The concentration of organic acids was measured according to the procedure described in Section 3.2.5.

7.2.6.4 Determination of oligosaccharides

The extraction of sugars from fermented and unfermented soymilk samples was performed using the method described previously by Scalabrini *et al*. (1998) with some modifications. Briefly, 3 mL aliquots were centrifuged at 14,000 x g for 30 min for protein removal, followed by filtration using a 0.20 μm membrane filter (Schleicher & Schuell, Dassel, Germany). The concentration of raffinose, stachyose and sucrose was determined with a Varian HPLC (Varian Analytical Instruments, CA, USA) fitted with an Alltima amino column (250 x 4.6 mm x 5 μm 100 Å) and corresponding guard column (Alltech Associates, Deerfield, IL, USA) maintained at 30^{o}C and an RI detector (ERC-7515A, ERMA Cr. Inc., Kawaguchi City, Japan). The mobile phase consisted of 75% acetonitrile and 25% distilled water and was maintained at a flow rate of 1 mL/min isocratically. A 20 μL injection volume was used for both samples and standards. The retention times of the standards for raffinose, stachyose and sucrose (Sigma) were at 11.4, 19.1 and 7.8 min, respectively. Standard stock solutions of raffinose (2.53 g/100 mL), stachyose (2.03 g/100 mL) and sucrose (2.53 g/100 mL) were used for preparation of standard calibration curve. The concentration of oligosaccharides was derived from the standard curve and was expressed as milligram of sugar per 100 mL of soymilk.

7.2.6.5 Proteolytic activity of cultures in fermented soymilk

Proteolysis during fermentation of soymilk was determined according to the procedure described in Section 3.2.6.

7.2.6.6 In vitro inhibition of angiotensin I-converting enzyme

ACE inhibitory activity was measured according to the procedure described in Section 4.2.6.

7.2.7 Statistical analysis

All results obtained were analysed as a split plot in time design using general linear model (GLM) procedure of the SAS System (SAS, 1996). The univariate ANOVA test was validated by fulfilling Huynh-Feldt (H-F) condition (Littell *et al*., 1998). Where appropriate, one-way ANOVA and correlational analysis were employed using Microsoft® Excel StatPro™ (Albright *et al*., 1999) and the multicomparison of means was assessed by Tukey's test. The statistical level of significance was preset at 0.05.

7.3 RESULTS AND DISCUSSIONS

7.3.1 α–Galactosidase specific activity of probiotic organisms and of *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus*

The α-galactosidase activity of the probiotic organisms and that of *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus* is shown in Table 7.1. The organisms exhibited α-galactosidase activity at varying degrees, and fermentation time was a significant ($P = 0.0007$) factor for α-galactosidase activity during the 48 h incubation at 37°C. This might be due to growth differences in the respective media which likely determined the amount of enzymes produced during fermentation. Interestingly, α-galactosidase activity in the medium containing raffinose declined substantially during 24 to 36 h of fermentation for some organisms but increased significantly ($P < 0.05$) for all microorganisms at 48 h. This followed the growth pattern of the organisms in the medium during the fermentation period. However, *B. lactis* B94 grew better than the other organisms in that medium as α-galactosidase activity increased significantly ($P < 0.05$) until the end of fermentation. This suggests that *B. lactis* B94 may have hydrolyzed raffinose mainly by producing acetate and lactate from the bifidus carbohydrate catabolism pathway. Scalabrini *et al*. (1998) also reported the production of α-galactosidase by *Bifidobacterium* strains in a modified medium. On the other hand, *L. delbrueckii* ssp. *bulgaricus* showed the lowest α-galactosidase activity in the medium containing raffinose (compared to other organisms). Although α-galactosidase specific activity in the glucose/raffinose medium was not as high as that of medium containing raffinose, the trend of activity was similar. The presence of glucose in MRS medium, on the other hand, resulted in high α-galactosidase activity for the majority of the organisms in comparison to other media except *B. lactis* B94 in 2% raffinose. All organisms grew well in MRS medium, which suggests that an increase in α-galactosidase is due to an increase in cell density. After reaching the log phase, α-galactosidase activity was reduced ($P < 0.05$). This reduction was most probably a result of depletion of the readily available carbon source or other factors such as accumulation of organic acids, drop in pH and reduction/aging bacteria population (Table 7.1).

The presence of 1% each of glucose and raffinose did not stimulate ($P > 0.05$) the synthesis of α-galactosidase as much as that containing 2% raffinose did (Table 7.1). Tsangalis and Shah (2004) reported that 1% raffinose supplementation increased the production of α-galactosidase for *Bifidobacterium* (BB536 and BP20099) strains but with little or no effect on the α-galactosidase activity of *Bifidobacterium* (BB12 and BL1941).

Since raffinose is an α-galactoside sugar found in soymilk, these selected organisms may grow well in soymilk for manufacture of soy based yoghurt.

Table 7.2 shows the effect of raffinose and glucose on the production of organic acids by probiotic organisms and by *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus* in supplemented media. *B. lactis* B94 exhibited the highest ($P < 0.05$) lactic and acetic acids production in 2% raffinose medium with significant ($P < 0.05$) increases during fermentation as opposed to other organisms which produced organic acids in comparable quantities ($P > 0.05$). On the other hand, *B. longum* Bl 536 produced low acetic acid in comparison to *B. lactis* B94. Scalabrini *et al*. (1998) reported that some *Bifidobacterium* strains produced relatively low quantities of acetic acid in a modified medium. The production of acetic acid in 1% raffinose and 1% glucose by *L. acidophilus* (La4962 and L10), *B. lactis* B94 and *B. longum* Bl 536, *L. casei* Lc279 and *L. casei* L26, *S. thermophilus* St1342 and *L. delbrueckii* ssp. *bulgaricus* Lb 1466 showed a slight ($P > 0.05$) variation in concentrations to that produced in the 2% raffinose. The mixture of glucose/raffinose stimulated higher ($P < 0.05$) production of lactic acid by all organisms studied compared to the medium containing 2% raffinose. The MRS broth which contained 2% glucose showed similar production of lactic acid to that of the mixture but low ($P < 0.05$) production of acetic acid for all the organisms (Table 7.2). Our study showed metabolism of raffinose as the sole energy source produced substantial amount of acetic acid than lactic acid.

7.3.2 Sugars metabolism in soymilk

Tables 7.3 and 7.4 show the utilization of raffinose, stachyose and sucrose and the production of lactic and acetic acids. The utilization of raffinose and stachyose in soymilk varied and appeared to depend on the α–galactosidase activity of the organism (Table 7.1). *S. thermophilus* St1342, *L. acidophilus* La 4962 and *B. lactis* B94 reduced raffinose significantly ($P < 0.05$) by 64.5%, 55.9% and 77.4%, respectively, whereas the remaining organisms showed less than 30% reduction after 48 h. Scalabrini *et al*. (1998) found that *Bifidobacterium* strains metabolized raffinose in soymilk, as opposed to yoghurt cultures, which did not reduce raffinose and stachyose during growth in soymilk. Our study was in line with that reported by Scalabrini *et al*. (1998), which showed that raffinose, was substantially metabolized by *Bifidobacterium* strains. The organisms in general metabolized stachyose by over 40% after 48 h, with *B. lactis* B94 and *L. acidophilus* L10 showing the highest hydrolysis of 63.5% and 57.5%, respectively. Our findings are in line with those of Mital and Steinkraus (1975), who reported that fermentation of soymilk with lactic cultures

possessing α-galactosidase activity reduced raffinose and stachyose contents. Overall, the sucrose concentration in soymilk was significantly ($P < 0.05$) reduced by *S. thermophilus* St1342, *B. lactis* B94 and *L. casei* Lc 279 after 48 h (Table 7.3).

7.3.3 Proteolytic activity

The proteolytic activity of the organisms in soymilk is presented in Figure 7.1. The amount of liberated amino groups and peptides increased only slightly ($P > 0.05$) during fermentation from 0 to 12 h for *L. acidophilus* La 4962, *B. longum* Bl 536, *B. lactis* B94, *L. casei* Lc 279 and *S. thermophilus* St1342, but increased significantly ($P < 0.05$) for the majority of the strains from 24 to 48 h (Figure 7.1). *L. delbrueckii* ssp. *bulgaricus* Lb 1466 showed the highest proteolytic activity ($P < 0.005$) in comparison to other organisms. *L. delbrueckii* ssp. *bulgaricus* has been considered as highly proteolytic in dairy systems (Abraham *et al*., 1993; Shihata and Shah, 2000). Our study showed that *L. delbrueckii* ssp. *bulgaricus* strains are also proteolytic in soymilk. *B. lactis* B94 was the next highest proteolytic organism in soymilk showing a significant ($P < 0.005$) production of free amino groups during 24 to 48 h of fermentation. The rest of the organisms exhibited varying degrees of proteolytic activity and all showed increasing proteolysis ($P < 0.005$) with time (Figure 7.1). The proteolytic activity in soymilk appeared to be strain specific ($P < 0.0001$) and also was time dependent ($P < 0.0001$). Other studies have also reported proteolytic activities with LAB in soy-based products (Omafuvbe *et al*., 2002). Strain specificity of proteolytic activity (Shihata and Shah, 2000; Fuglsang *et al*., 2003) is not confined to bovine milk medium only but is also exhibited in soymilk as was observed in Chapter 4.

Some amino acids and peptides were used by the organisms for cell growth and survival (Nielsen *et al*., 2001), therefore high proteolytic activity of these organisms in soymilk may have contributed to appreciable cell growth in addition to their ability to metabolize stachyose and raffinose as energy sources. The correlation between proteolytic activity and growth, which ranged from 0.61 for *S. thermophilus* St 1342 to 0.98 for *L. casei* Lc 279, further indicate the substantial effect of proteolytic activity on bacterial growth. The influence of proteolytic activity on cell growth of *Bifidobacterium* sp. and some selected probiotic organisms as well as *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus* in soymilk was reported in Chapter 4 and also by Kamaly (1997).

7.3.4 Viability of selected microorganisms and organic acids production in fermented soymilk

In general, probiotic cultures achieved the desired therapeutic level (10^8 cfu/mL) during growth by each organism in soymilk after 48 h of fermentation as shown in Table 7.4. Organic acid production, pH decline and other metabolic activities occurred during the first 12 to 24 h of incubation, which corresponded to the exponential phase of the growth. *B. longum* Bl 536 and *L. casei* L26 exhibited better growth ($P < 0.05$) throughout the cultivation compared to other microorganisms. On the other hand, *L. acidophilus* L10 and *L. delbrueckii* ssp. *bulgaricus* Lb1466 showed slow growth by one log cycle lower ($P < 0.05$) than the rest of the organisms throughout the incubation (Table 7.4). *S. thermophilus* St 1342 grew better than *L. delbrueckii* ssp. *bulgaricus* Lb1466 and produced higher ($P < 0.05$) organic acid than the latter during fermentation. Wang *et al*. (1974) reported that *L. delbrueckii* ssp. *bulgaricus* grew poorly in soymilk because of their inability to ferment sucrose and other soy carbohydrates. On the other hand, it has been demonstrated in Chapter 4 that *S. thermophilus* St 1342 and *L. delbrueckii* ssp. *bulgaricus* Lb1466 grew well as a mixed culture in soy yoghurt. This was evident by the low ($P < 0.05$) α-galactosidase activity of *L. delbrueckii* ssp. *bulgaricus* Lb1466 (Table 7.1). Mital *et al*. (1974) also reported that certain organisms such as *S. thermophilus*, *L. acidophilus, L. cellobiosis* and *L. plantarum,* which utilized sucrose, grew well and produced large amounts of acid in soymilk. However contrary to our finding, the selected organisms used in this study produced lower amounts of organic acids in soymilk eventhough they grew well. Liu (1997) also reported that LAB grew well in soymilk but produce less organic acids. The low levels of organic acid concentrations in fermenting soymilk presumably encouraged cell growth as noted in Chapter 4 and in other studies (Angeles and Marth, 1971; Kamaly, 1997; Liu, 1997). Furthermore, these selected strains possess α-galactosidase (Table 7.1) and can utilize sucrose and other soy carbohydrates in soymilk as sources of energy, which enhanced better cell growth during 48 h fermentation at 42°C (Mital *et al*., 1974; Liu, 1997). Although the organisms in our study showed a consistent increase in cell concentration from the start of incubation until 24 h, the required pH of 4.5 was not reached within this time frame in comparison to the MRS medium with the exception of *L. casei* L26 that showed a pH decline of 4.12 (Table 7.4). However, after a prolonged incubation for 48 h, the pH of all batches declined to below 4.5. In general, the production of organic acids by all organisms in MRS was significantly higher ($P < 0.05$) than those produced in the fermented soymilk from 0 to 48 h (Tables 7.2 and 7.4), thus making soymilk a potential medium for bacterial

growth. Soymilk was reported previously as an appropriate growth medium for some lactic acid bacteria (Angeles and Marth, 1971; Kamaly, 1997; Liu, 1997).

7.3.5 ACE-inhibitory activity

The ACE inhibitory activity of all organisms studied is shown in Figure 7.2. The percentage inhibition values between the bacterial strains varied ($P < 0.05$), indicating possible differences in ACE-inhibitory peptides produced by the organisms. In general, ACE inhibitory activity appeared to be the highest at 24 h for all organisms but the overall inhibition was below 50%. *B. longum* Bl 536 showed highest activity (43.4%) at 48 h as opposed to other organisms. *L. acidophilus* strains were not as proteolytic as *L. delbrueckii* ssp. *bulgaricus* Lb1466, *B. lactis* B94 and *L. casei* L26 but showed appreciable ACE inhibitory activity. Several studies have shown that soy protein hydrolysates, obtained mainly by alkaline hydrolysis, have lowered high blood pressure in hypertensive rats (Wu and Ding 2002; Yang *et al*., 2004). The results showed that the production of ACE inhibitory activity is strain specific, however, not all released peptides in fermented soymilk as a result of proteolysis may express *in vitro* ACE-inhibition.

7.4 CONCLUSIONS

L. acidophilus (La4962 and L10), *B. lactis* B94 and *B. longum* Bl 536, *L. casei* Lc279 and *L. casei* L26, *S. thermophilus* St1342 and *L. delbrueckii* ssp. *bulgaricus* Lb 1466 exhibited variable α-galactosidase activity with *B. lactis* B94 showing the highest activity in MRS supplemented media. However, all organisms reached the desired therapeutic level (10^8 cfu/mL) likely due to their ability to metabolize oligosaccharides during fermentation in soymilk at 42°C. The oligosaccharide metabolism depended on α-galactosidase activity. *B. lactis* B94, *S. thermophilus* St1342 and *L. acidophilus* La 4962 reduced raffinose by 77.4, 64.5 and 55.9%, respectively in soymilk. The organisms exhibited varying proteolytic activity and all showed increasing proteolysis with time. As a result of proteolytic activity, peptides released showed ACE-inhibitory activity, which appeared to depend on strain. Thus fermented soymilk could be converted into a rich functional product containing probiotics and bioactive compounds.

Table 7.1 α-Galactosidase activity of probiotic and yoghurt cultures in supplemented media at 37°C for 48 h

Strain	Time, h	α-galactosidase specific activity* U/mg		
		Raffinose	Glucose/ Raffinose	Glucose
L. delbruekii ssp. *bulgaricus* Lb1466	12	7.02 ^{a}	9.35 ^{a}	14.82 ^{a}
	24	5.18 ^{a}	7.69 ^{a}	12.65 ^{a}
	36	5.57^{a}	6.90 ^{ab}	14.37 ^{a}
	48	6.72 ^{a}	17.60 ^{bc}	14.58 ^{a}
S. thermophilus St1342	12	11.32 ^{a}	9.60 ^{a}	34.13 ^{a}
	24	9.68 ^{a}	6.03 ^{b}	13.81 ^{b}
	36	7.40 ^{a}	2.79 ^{c}	13.85 ^{b}
	48	15.05 ^{ab}	13.10 ^{d}	11.25 ^{b}
L. acidophilus L10	12	10.21 ^{Aa}	9.22 ^{Aa}	18.00 ^{Aa}
	24	9.12 ^{Aa}	3.49 ^{Cb}	13.01 ^{Cb}
	36	10.24 ^{Aa}	4.28 ^{Db}	11.84 ^{Db}
	48	14.86 ^{ABa}	9.87 ^{Ea}	11.93 ^{Eb}
L. acidophilus La 4962	12	9.38 ^{Aa}	7.23 ^{Ba}	42.09 ^{Ba}
	24	7.78 ^{Aa}	4.70 ^{BCDb}	21.40 ^{Bb}
	36	4.02 ^{CAa}	5.46 ^{BDab}	10.79 ^{BCDEc}
	48	18.94 ^{Bb}	8.91 ^{EAac}	28.85 ^{Bd}
B. lactis B94	12	11.07 ^{Aa}	7.64 ^{Aa}	41.88 ^{Aa}
	24	25.05 ^{Aa}	4.57 ^{Ca}	34.34 ^{Cb}
	36	155.40 ^{Ab}	3.94 ^{Da}	24.45 ^{Bbc}
	48	183.29 ^{Cb}	11.05 ^{Eab}	14.45 ^{Ebcd}
B. longum Bl 536	12	14.56 ^{ABab}	22.05 ^{Ba}	23.33 ^{Ba}
	24	10.68 ^{ABa}	9.13 ^{AEb}	28.37 ^{Ba}
	36	8.68 ^{ABa}	10.04 ^{AEbc}	12.25 ^{BDEb}
	48	19.11 ^{ABa}	9.74 ^{AEbcd}	20.95 ^{Bac}
L. casei L26	12	16.71 ^{Aa}	9.21 ^{Aa}	17.98 ^{Aa}
	24	8.19 ^{ABb}	10.80 ^{Aa}	35.65 ^{Bb}
	36	15.08 ^{Ca}	5.98 ^{Cb}	7.70 ^{Cabc}
	48	18.85 ^{Da}	13.79 ^{Db}	9.97 ^{Ea}
L. casei Lc 279	12	6.02 ^{Ba}	9.21 ^{Aa}	27.39 ^{Ba}
	24	8.42 ^{Ba}	3.78 ^{Bb}	30.71 ^{Ba}
	36	6.81 ^{Ba}	5.92 ^{BCbc}	22.25 ^{Aa}
	48	14.00 ^{CAa}	9.82 ^{Aa}	10.79 ^{ACEb}
SEM		6.81		

^{abcd} Means in the same column for a particular strain with different small letter superscripts are significantly different; ^{ABCDE} Means in the same column for particular strains with different capital letter superscripts are significantly different. One unit of enzyme was defined as the amount of enzyme required to release 1nmol of p-ntrophenol from p-nitrophenyl-α-D-galactopyranoside per mL per min under assay conditions. Results presented as a mean of three observations; SEM - pooled standard error of the mean (0.03); the level of significance was preset at p = 0.05.

Table 7.2 Production of organic acids by selected probiotic and yoghurt cultures in supplemented MRS at 37°C for 48 h

Strain	Time, h	Organic acids, mg/mL					
		Raffinose		Glucose/Raffinose		Glucose	
		LA#	AA*	LA	AA	LA	AA
L. delbruekii ssp. *bulgaricus* Lb1466	12	1.77 ^{a}	4.28 ^{a}	9.89 ^{a}	4.46 ^{a}	8.47 ^{a}	2.27 ^{a}
	24	1.51 ^{a}	5.76 ^{ba}	9.96 ^{a}	4.04 ^{a}	12.08 ^{b}	2.24 ^{a}
	36	1.67 ^{a}	5.60 ^{ab}	11.35 ^{a}	5.03 ^{a}	13.16 ^{b}	2.23 ^{a}
	48	1.57 ^{a}	5.87 ^{b}	10.54 ^{a}	4.51 ^{a}	13.69 ^{b}	2.34 ^{a}
S. thermophilus	12	1.66 ^{a}	4.90 ^{a}	10.36 ^{a}	4.56 ^{a}	8.42 ^{a}	2.19 ^{a}
	24	1.64 ^{a}	5.78 ^{a}	11.42 ^{a}	6.49 ^{b}	11.19 ^{b}	2.28 ^{a}
	36	1.67 ^{a}	5.75 ^{a}	10.49 ^{a}	5.66 ^{ab}	13.16 ^{b}	2.22 ^{a}
	48	1.69 ^{a}	6.15 ^{a}	10.64 ^{a}	3.79 ^{a}	13.51 ^{b}	2.23 ^{a}
L. acidophilus L10	12	1.40 ^{Aa}	4.08 ^{Aa}	11.83 ^{Aa}	6.62 ^{Aa}	9.93 ^{Aa}	2.45 ^{Aa}
	24	1.03 ^{Ba}	4.32 ^{BAa}	11.83 ^{Aa}	7.54 ^{Ba}	13.45 ^{BCb}	2.51 ^{Aa}
	36	1.36 ^{Aa}	5.04 ^{Ba}	10.41 ^{Aa}	6.44 ^{Aa}	13.57 ^{BDb}	2.69 ^{Aa}
	48	1.11 ^{Aa}	4.50 ^{Ba}	10.39 ^{Aa}	5.92 ^{Aa}	13.63 ^{BEb}	2.81 ^{Ca}
L. acidophilus La 4962	12	1.57 ^{ACa}	5.86 ^{Ba}	10.28 ^{Aa}	4.52 ^{ACa}	9.31 ^{Aa}	2.26 ^{Aa}
	24	1.48 ^{Aa}	5.77 ^{Ba}	11.49 ^{Aa}	6.39 ^{Aa}	13.02 ^{Bb}	2.26 ^{Aa}
	36	1.53 ^{Aa}	5.69 ^{Ba}	11.20 ^{Aa}	6.77 ^{Aa}	13.25 ^{Bb}	1.93 ^{Ba}
	48	1.51 ^{Aa}	5.66 ^{Ba}	10.25 ^{Aa}	4.62 ^{ABCa}	13.30 ^{Bb}	2.26 ^{Aa}
B. lactis B94	12	1.54 ^{Aa}	6.14 ^{Aa}	10.38 ^{Aa}	4.86 ^{Aa}	9.20 ^{Aa}	2.46 ^{Aa}
	24	2.16 ^{Aa}	5.96 ^{Aa}	11.23 ^{Aa}	6.61 ^{Aa}	13.01 ^{BCb}	2.28 ^{Aa}
	36	5.02 ^{Bab}	8.07 ^{Aab}	11.27 ^{Aa}	6.52 ^{Aa}	13.58 ^{BDb}	2.30 ^{Aa}
	48	6.34 ^{Db}	12.84 ^{Bb}	9.36 ^{Aa}	3.65 ^{ABab}	13.12 ^{BEb}	2.30 ^{Aa}
B. longum Bl 536	12	2.62 ^{ABa}	3.92 ^{ACa}	10.33 ^{Aa}	4.69 ^{ABa}	9.31 ^{Aa}	2.20 ^{Aa}
	24	1.24 ^{ACa}	5.92 ^{ACa}	11.41 ^{Aa}	5.74 ^{ABa}	12.90 ^{Bb}	2.28 ^{Aa}
	36	1.70 ^{ACa}	5.40 ^{ACa}	11.32 ^{Aa}	6.79 ^{ACa}	13.54 ^{Bb}	2.32 ^{Aa}
	48	1.25 ^{ACa}	5.69 ^{ACa}	10.35 ^{Aa}	4.48 ^{ABa}	13.51 ^{Bb}	2.35 ^{Aa}
L. casei L26	12	1.41 ^{Aa}	5.24 ^{Aa}	10.64 ^{Aa}	4.98 ^{Aa}	8.10 ^{Aa}	2.24 ^{Aa}
	24	1.12 ^{Aa}	5.25 ^{Aa}	12.23 ^{Aa}	7.07 ^{Bab}	11.74 ^{BCb}	2.29 ^{Aa}
	36	1.39 ^{Aa}	5.32 ^{Aa}	11.07 ^{Aa}	4.07 ^{ACabc}	13.56 ^{BDb}	2.30 ^{Aa}
	48	1.21 ^{Aa}	5.35 ^{Aa}	12.06 ^{Aa}	5.52 ^{ACab}	13.53 ^{BEb}	2.25 ^{Aa}
L. casei Lc 279	12	1.57 ^{Aa}	5.51 ^{Aa}	9.40 ^{Aa}	3.53 ^{ACa}	9.02 ^{Aa}	2.08 ^{Aa}
	24	1.32 ^{Aa}	5.70 ^{Aa}	9.83 ^{Aa}	3.53 ^{ACa}	13.38 ^{Bb}	2.29 ^{Aa}
	36	1.62 ^{Aa}	5.79 ^{Aa}	9.81 ^{Aa}	4.12 ^{ACa}	13.55 ^{Bb}	2.34 ^{Aa}
	48	1.19 ^{Aa}	4.65 ^{Aa}	10.32 ^{Aa}	4.56 ^{ACa}	13.65 ^{Bb}	2.30 ^{Aa}
SEM		0.49	0.35	0.49	0.35	0.49	0.35

#LA = lactic acid; *AA = Acetic acid; ^{abc} Means in the same column for a particular strain with different small letter superscripts are significantly different; ^{ABCDE} Means in the same column for particular strains with different capital letter superscripts are significantly different. Results presented as a mean of three observations. Significant when $P < 0.05$; SEM - pooled standard error of the mean (0.49 and 0.35).

Table 7.3 Changes in concentration of raffinose, stachyose and sucrose in soymilk during fermentation with probiotic organisms and yoghurt culture for 0, 6, 12, 24, and 48 h at 42°C

Sugar/strain	Sugar concentration, mg /100 mL				
	Time, h				
	0	6	12	24	48
Raffinose					
Lb1466	3.49 ^{a}	3.07 ^{a}	2.91 ^{a}	2.78 ^{a}	2.69 ^{Aa}
St1342	3.49 ^{a}	2.73 ^{a}	2.55 ^{a}	2.38 ^{a}	1.24 ^{Aa}
L10	3.49 ^{a}	3.33 ^{Aa}	3.21 ^{Aa}	3.20 ^{Aa}	2.51 ^{Aa}
La 4962	3.49 ^{a}	2.51 ^{Aa}	2.23 ^{Ba}	1.81 ^{Ba}	1.54 ^{Aa}
B94	3.49 ^{a}	2.50 ^{Aab}	2.19 ^{Aab}	1.21 ^{Ab}	0.79 ^{Ab}
Bl 536	3.49 ^{a}	3.43 ^{Ba}	3.15 ^{Ba}	2.76 ^{Ba}	2.63 ^{Ba}
L26	3.49 ^{a}	3.45 ^{Aa}	2.95 ^{Aa}	2.87 ^{Aa}	2.82 ^{Aa}
Lc 279	3.49 ^{a}	3.43 ^{Aa}	3.21 ^{Aa}	2.59 ^{Aa}	2.71 ^{Aa}
SEM			0.52		
Stachyose					
Lb1466	14.13 ^{a}	8.99 ^{a}	8.64 ^{a}	8.48 ^{a}	7.90 ^{Aa}
St1342	14.13 ^{a}	13.78 ^{a}	10.74 ^{a}	10.22 ^{a}	8.38 ^{Aa}
L10	14.13 ^{a}	10.34 ^{Aa}	9.74 ^{Aa}	7.40 ^{Aa}	6.00 ^{Aa}
La 4962	14.13 ^{a}	12.08 ^{Ba}	11.00 ^{Ba}	10.07 ^{Ba}	8.43 ^{Aa}
B94	14.13 ^{a}	11.24 ^{Aab}	9.81 ^{Aab}	7.85 ^{Aab}	5.16 ^{Ab}
Bl 536	14.13 ^{a}	13.90 ^{Ba}	12.79 ^{Ba}	12.84 ^{Ba}	11.11 ^{Ba}
L26	14.13 ^{a}	12.98 ^{Aa}	11.04 ^{Aa}	9.62 ^{Aa}	8.22 ^{Aa}
Lc 279	14.13 ^{a}	12.20 ^{Ba}	10.94 ^{Aa}	9.22 ^{Ab}	9.20 ^{Bb}
SEM			3.23		
Sucrose					
Lb1466	202.00 ^{a}	187.57 ^{a}	176.70 ^{a}	131.45 ^{a}	123.03 ^{Aa}
St1342	202.00 ^{a}	201.11 ^{ab}	164.11 ^{ab}	158.12 ^{ab}	87.67 ^{Bb}
L10	202.00 ^{a}	196.77 ^{Aa}	193.93 ^{Aa}	136.28 ^{Aa}	115.77 ^{Aa}
La 4962	202.00 ^{a}	174.53 ^{Ba}	168.40 ^{Ba}	159.69 ^{Ba}	148.50 ^{Ba}
B94	202.00 ^{a}	164.31 ^{Aab}	160.49 ^{Aab}	123.72 ^{Aab}	83.54 ^{Ab}
Bl 536	202.00 ^{a}	196.09 ^{Bab}	141.51 ^{Bab}	138.33 ^{Bab}	113.19 ^{Bb}
L26	202.00 ^{a}	175.74 ^{Aa}	116.02 ^{Aa}	109.58 ^{Aa}	102.55 ^{Aa}
Lc 279	202.00 ^{a}	161.01 ^{Bb}	138.45 ^{Bc}	109.09 ^{Ad}	77.03 ^{Be}
SEM			15.21		

Lb1466 = *L. delbrueckii* ssp. *bulgaricus*; St1342 = *S. thermophilus*; *L10* & La 4962 = *L. acidophilus*; B94 = *B. lactis*; Bl 536 = *B. longum*; L26 = *L. casei*; Lc 279 = *L. casei*. ^{abcde} Means in the same column for a particular strain with different small letter superscripts are significantly different; ^{AB} Means in the same column for particular strains with different capital letter superscripts are significantly different. Results presented as a mean of three observations. Significant when $P < 0.05$; SEM - pooled standard error of the mean (0.52, 3.23 and 15.21).

Table 7.4 Metabolic activity of investigated cultures during growth in soymilk for 48 h at 42°C

Strain	Time h	pH	Cell counts, cfu/mL	Organic acids, mg/mL	
				Lactic	Acetic
L. delbrueckii ssp. *bulgaricus* Lb1466	0	6.67 ^{a}	6.69 ^{a}	0.00 ^{a}	0.00 ^{a}
	6	6.53 ^{b}	7.21 ^{a}	0.01 ^{a}	0.00 ^{a}
	12	6.45 ^{b}	7.43 ^{ab}	0.01 ^{ab}	0.02 ^{a}
	24	5.82 ^{bc}	7.82 ^{b}	0.03 ^{b}	0.06 ^{b}
	48	5.59 ^{abcd}	7.88 ^{b}	0.04 ^{b}	0.07 ^{b}
S. thermophilus St1342	0	6.66 ^{a}	7.44 ^{a}	0.01 ^{a}	0.00 ^{a}
	6	6.50 ^{b}	7.59 ^{a}	0.01 ^{a}	0.02 ^{b}
	12	6.39 ^{c}	8.04 ^{b}	0.02 ^{b}	0.06 ^{bc}
	24	4.90 ^{d}	8.23 ^{b}	0.17 ^{bc}	0.07 ^{bcd}
	48	4.00 ^{e}	8.24 ^{b}	0.33 ^{bcd}	0.07 ^{bcd}
L. acidophilus L10	0	6.64 ^{Aa}	6.66 ^{Aa}	0.01 ^{Aa}	0.00 ^{Aa}
	6	6.46 ^{Aab}	6.94 ^{Cab}	0.01 ^{Aa}	0.00 ^{Aa}
	12	6.16 ^{Bb}	7.30 ^{Bb}	0.02 ^{Aa}	0.00 ^{Aa}
	24	4.93 ^{Dbc}	7.30 ^{Bb}	0.09 ^{Aa}	0.01 ^{Aa}
	48	4.10 ^{Ebcd}	7.37 ^{Bb}	0.26 ^{Bb}	0.03 ^{Aa}
L. acidophilus La 4962	0	6.66 ^{ACa}	7.67 ^{Ba}	0.02 ^{Aa}	0.00 ^{Aa}
	6	6.50 ^{ABa}	7.71 ^{Ba}	0.01 ^{Aa}	0.03 ^{ABb}
	12	6.44 ^{ABa}	8.08 ^{BDab}	0.02 ^{Aa}	0.05 ^{Bb}
	24	6.41 ^{ABa}	8.17 ^{BDb}	0.04 ^{Aa}	0.06 ^{Bbc}
	48	4.14 ^{BCEb}	8.81 ^{BDbc}	0.28 ^{Bb}	0.07 ^{Bbc}
B. lactis B94	0	6.62 ^{Aa}	7.25 ^{Aa}	0.00 ^{Aa}	0.00 ^{Aa}
	6	6.45 ^{Aa}	7.50 ^{Aab}	0.01 ^{Aa}	0.02 ^{Aa}
	12	6.43 ^{Aa}	7.86 ^{ABab}	0.02 ^{Aa}	0.05 ^{Bb}
	24	4.89 ^{Cb}	7.96 ^{ABCab}	0.09 ^{Aab}	0.11 ^{Cbc}
	48	4.42 ^{Cb}	8.44 ^{Eb}	0.19 ^{Cb}	0.12 ^{Cbc}
B. longum Bl 536	0	6.60 ^{Aa}	7.24 ^{Aa}	0.01 ^{Aa}	0.01 ^{Aa}
	6	6.40 ^{Aa}	8.03 ^{AEab}	0.02 ^{Aa}	0.02 ^{ABa}
	12	6.22 ^{Aa}	8.82 ^{BEbc}	0.03 ^{Aa}	0.05 ^{Bba}
	24	4.55 ^{CBb}	8.84 ^{BCEbc}	0.21 ^{ABCb}	0.06 ^{Bba}
	48	3.81 ^{Bbc}	9.54 ^{BCDbc}	0.40 ^{Bbc}	0.06 ^{Bba}
L. casei L26	0	6.62 ^{Aa}	7.49 ^{Aa}	0.01 ^{Aa}	0.00 ^{Aa}
	6	6.36 ^{Aa}	8.81 ^{Cb}	0.02 ^{Aa}	0.03 ^{Cbc}
	12	6.13 ^{Cab}	8.26 ^{Dbc}	0.04 ^{Aa}	0.06 ^{Db}
	24	4.12 ^{Ebc}	8.79 ^{Ebd}	0.29 ^{Bb}	0.06 ^{Db}
	48	3.84 ^{Ebc}	9.13 ^{Fbd}	0.37 ^{Bbc}	0.05 ^{Db}
L. casei Lc 279	0	6.62 ^{ADa}	7.29 ^{Aa}	0.01 ^{Aa}	0.01 ^{Aa}
	6	6.41 ^{ACa}	7.68 ^{Aab}	0.01 ^{Aa}	0.02 ^{BCbc}
	12	6.38 ^{ACa}	7.94 ^{BDbc}	0.02 ^{Aa}	0.05 ^{BDb}
	24	6.39 ^{ACa}	8.16 ^{BDbcd}	0.03 ^{Aa}	0.06 ^{BDb}
	48	3.97 ^{BDEb}	8.88 ^{BCEFbc}	0.33 ^{Bb}	0.05 ^{BDb}
SEM		0.05	0.10	0.01	0.00

^{abcde} Means in the same column for a particular strain with different small letter superscripts are significantly different; ^{ABCDEF} Means in the same column for particular strains with different capital letter superscripts are significantly different. Results presented as a mean of three observations. Significant when $P < 0.05$; SEM - pooled standard error of the mean (0.05 and 0.10).

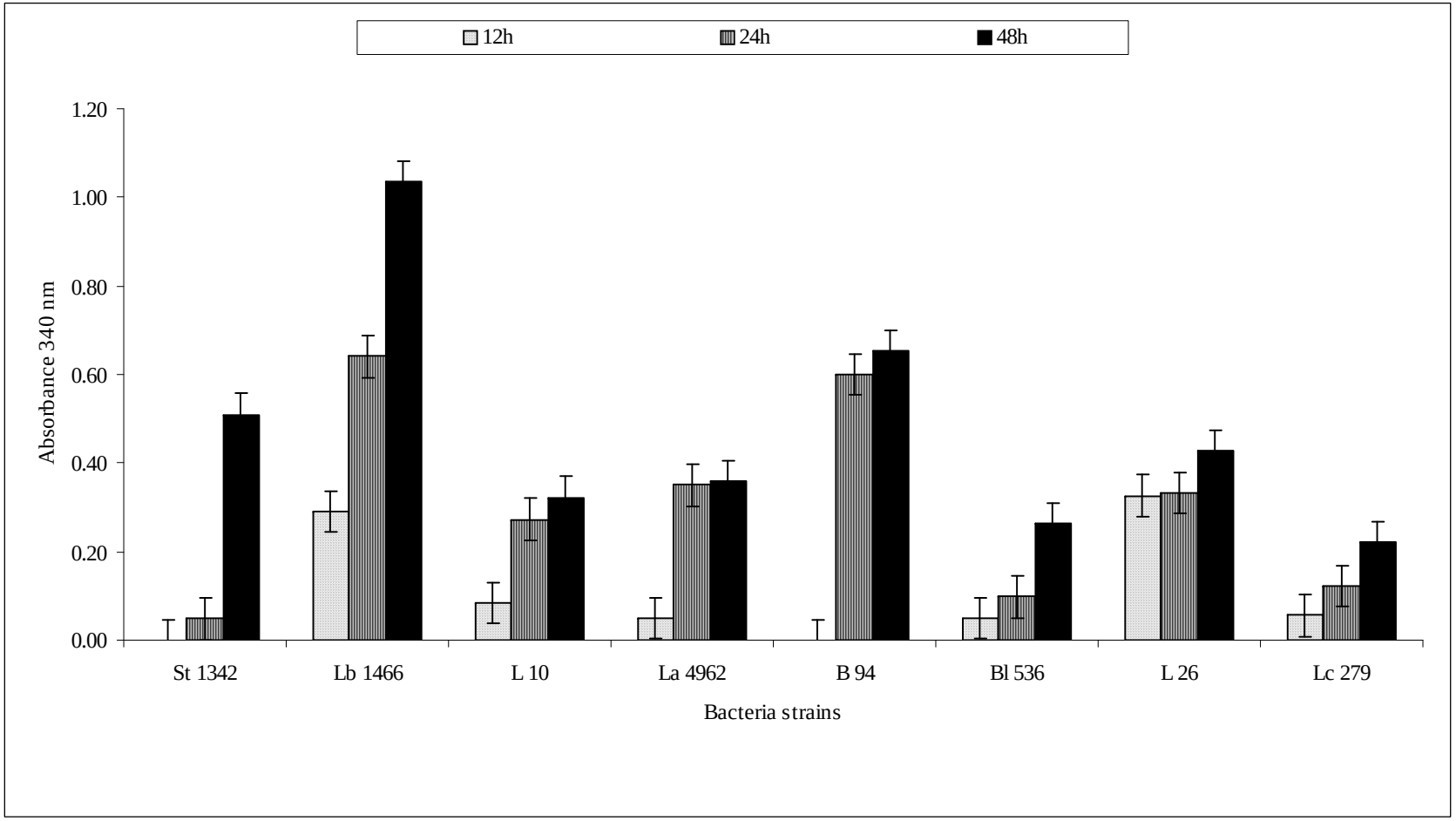

Figure 7.1 The extent of proteolysis in soymilk fermented with probiotic strains (*L. acidophilus* L10, *L. acidophilus* La 4962, *B. lactis* B94, *B. longum* Bl 536, *L. casei* L26 and *L. casei* Lc 279) and yoghurt culture (*S. thermophilus* St1342, and *L. delbrueckii* ssp. *bulgaricus* Lb 1466) for 12, 24, and 48 h at 42^{o}C. (Error bars represent a pooled standard error of the mean; SEM = 0.14 absorbance units).

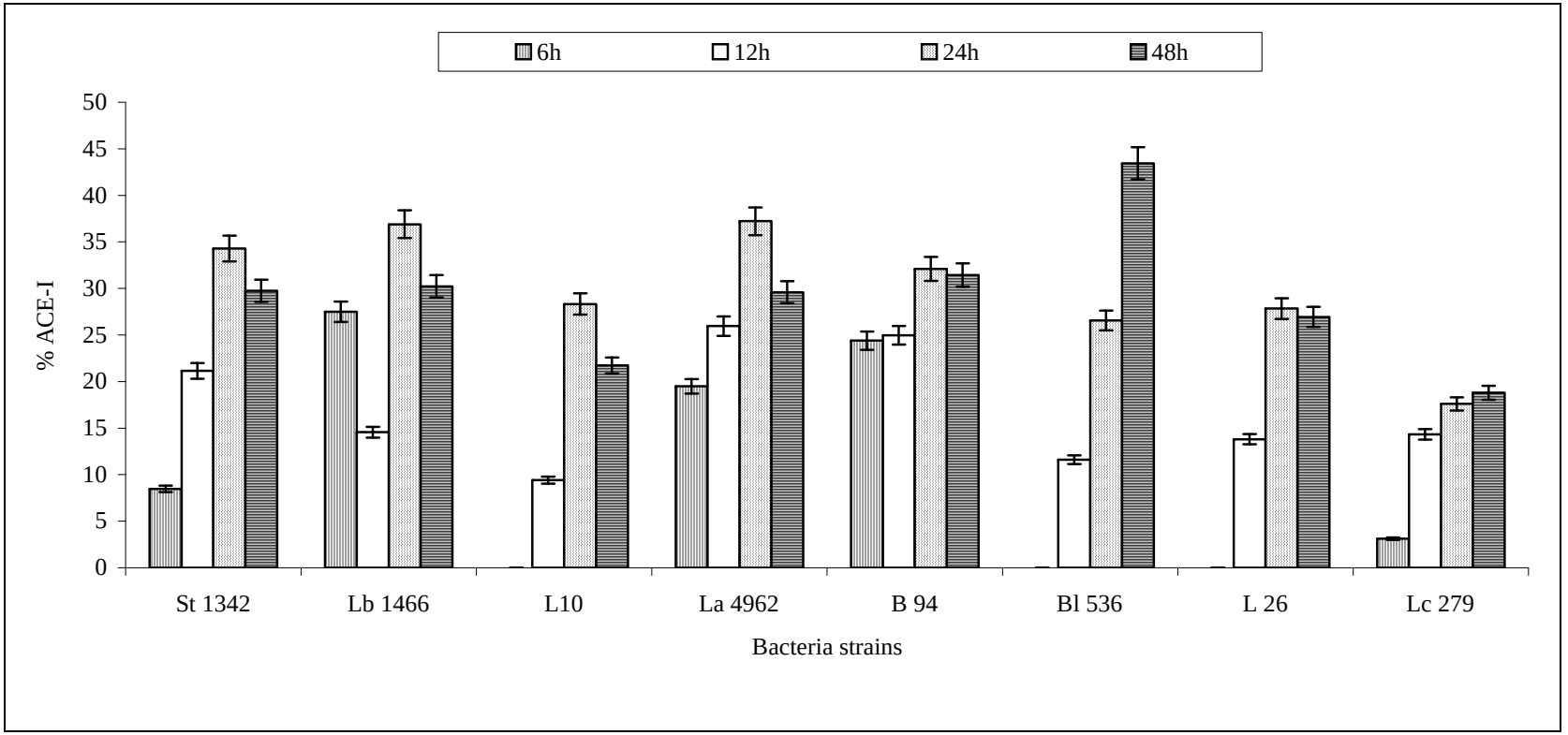

Figure 7.2 Angiotensin I-converting enzyme (ACE) inhibitory activity of fermented soymilk extracts obtained from individual culture (*S. thermophilus* St1342, *L. delbrueckii* ssp. *bulgaricus* Lb 1466, *L. acidophilus* L10, *L. acidophilus* La 4962, *B. lactis* B94, *B. longum* Bl 536, *L. casei* L26 and *L. casei* Lc 279) fermentations in soymilk after 6, 12, 24 and 48 h at 42^{o}C. (Error bars represent a pooled standard error of the mean; SEM = 3.87).

8.0 ACE-inhibitory activity of probiotic yoghurt

8.1 INTRODUCTION

Fermented milk products, in addition to providing both energy and nutrients, are an excellent source of bioactive peptides. Numerous peptides with bioactive properties have been isolated from fermented dairy products. These include antibacterial (Minervini *et al*., 2003), anticancer, immunomodulatory, mineral-binding, opioid (Shah, 2000a; Silva and Malcata, 2005) and antihypertensive (Muguerza *et al*., 2006) peptides. Among various bioactive peptides, the antihypertensive peptides or angiotensin converting enzyme inhibitors (ACE-I) are the most widely studied (Muguerza *et al*., 2006). ACE inhibition leads to a decrease in the level of the vasoconstricting peptide, angiotensin II, and a corresponding increase in the level of the vasodilatory peptide, bradykinin, and therefore yielding an overall reduction in blood pressure. Peptides with ACE-I activity have already been isolated from different food proteins (Ariyoshi, 1993; Yamamoto, 1997). These peptides have also been isolated from a variety of fermented dairy products including cheese, fermented milk and yoghurt (Fitzgerald and Murray, 2006). These biologically active peptides could represent a healthier and natural alternative for the ACE-I drugs.

In addition to the formation of lactic acid and flavour compounds, proteolysis is the most important biochemical process occurring in sour milk products during fermentation and storage (Tamime and Deeth, 1980). Extracellular proteinases are involved in the initial degradation of caseins, yielding a large number of oligopeptides. Consequently, further breakdown by intracellular peptidases is critical to fulfill the needs for essential and growth-stimulating amino acids and peptides (Kunji *et al*., 1996; Christensen *et al*., 1999). This proteolytic activity results in the release of bioactive peptides from specific amino acid sequences within the parent proteins and can provide physiological benefits (Pihlanto-Leppala *et al*., 1998; Gobbetti *et al*., 2000; Yoshikawa *et al*., 2000). The size of bioactive peptides may vary from two to twenty amino acid residues with their activity depending on the amino acid composition (Meisel and Fitzgerald, 2003; Korhonen and Pihlanto, 2006). Some low molecular weight antihypertensive peptides, such as Val-Pro-Pro (VPP) and Ile-Pro-Pro (IPP), resisted *in vivo* degradation by gastrointestinal enzymes and were able to exert antihypertensive activity through the inhibition of ACE in aorta (Nakamura *et al*., 1996; Nakamura *et al*., 1995). In a human feeding trial, sour milk containing VPP and IPP was effective in reducing the blood pressure of hypertensive patients. A product containing

Donkor, O. N., Henriksson, A., Singh, T. K., Vasiljevic, T., & Shah, N. P. (2007). ACE-inhibitory activity of probiotic yoghurt. *International Dairy Journal, 17*, 1321-1331.

VPP and IPP has since been commercialized (Seppo *et al*., 2003). In contrast to the vast amount of information that is available on production and characterization of antihypertensive peptides using enzymatic hydrolysis (Roy *et al*., 2000) or fermentation of milk with different microorganisms (Yamamoto *et al*., (1994a, 1994b); Takano, 1998; Gobbetti *et al*., 2002; Leclerc *et al*., 2002; Seppo *et al*., 2003; Pan *et al*., 2005), there is a very limited information on the profile of ACE-I activity in yoghurt containing selected probiotic cultures during cold storage. The selected microorganisms used in this study have been investigated extensively and shown to possess probiotic properties (Crittenden *et al*., 2005). Therefore, in addition to the claimed health benefits of probiotics (Lourens-Hattingh and Viljoen, 2001), it will be an added advantage if these selected microorganisms could produce bioactive peptides particularly ACE-I activity in yoghurt.

The objectives of this study were to investigate the release of ACE-I peptides in yoghurt using selected probiotic organisms, to examine the ACE-I profile of yoghurt during 28 d storage at 4^{o}C, and characterize isolated peptides by amino acid sequence analysis.

8.2 MATERIALS AND METHODS

8.2.1 Substrates and Chemicals

Hippuryl-histidyl-leucine, de Man Rogosa and Sharpe (MRS) bacteriological medium, o-phthaldialdehyde (OPA), trichloroacetic acid (TCA), sorbitol, bacteriological agar, nalidixic acid, neomycin sulphate, lithium chloride, paramomycin sulphate (NNLP), trifluoroacetic acid (TFA), L-cysteine.HCl, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), β-mercarptoethanol, Tris-HCl, glycerol, dithiothreitol, bromophenol blue, α-, β- casein, methanol and acetic acid were purchased from Sigma Chemical Company (St Louis, MO USA). Coomassie Brilliant blue (ICN Biochemicals Inc., Aurora, OH, USA), Skim milk powder was purchased from Murray Goulburn Co-operarative Co. Ltd. (Brunswick, Vic Australia). Acetonitrile, and reinforced clostridia agar (RCA) and bacteriological peptone were purchased from Merck (Darmstadt, Germany), and Oxoid (West Heidleberg, Australia), respectively.

8.2.2. Propagation of cultures

S. thermophilus St1342 and *L. delbrueckii* ssp. *bulgaricus* Lb1466 were obtained from the Victoria University Culture Collection (Werribee, Australia). *L. acidophilus* LAFTI® L10, *L. casei* LAFTI® L26 and *B. lactis* LAFTI® B94 were provided by DSM Food

Specialties (Moorebank, NSW, Australia). The organisms were stored at -80°C. Sterile 10 mL aliquots of MRS were inoculated with 1% of each organism and incubated at 42°C for *L. delbrueckii* ssp. *bulgaricus* Lb1466 and 37°C for *S. thermophilus* St1342, *L. acidophilus* LAFTI® L10, *L. casei* LAFTI® L26 and *B. lactis* LAFTI® B94. For propagation of *Bifidobacterium*, sterile MRS broth was supplemented with 0.05% L-cysteine.HCl in order to provide anaerobic condition and stimulate their growth (Ravula and Shah, 1998). Each activated microorganism was inoculated at 1% (v/v) into 10 mL aliquots of reconstituted skim milk (RSM, 12% w/w) supplemented with 2% glucose and 1% yeast extract. After two successive transfers the cultures were finally transferred into sterile RSM to obtain approximately 10^8 colony forming units (cfu) per millilitre for yoghurt manufacture. The inoculated milk was incubated at 37°C for 20 h. The last step was to ensure that no yeast extract was carried over during yoghurt manufacture which would interfere with readings.

8.2.3 Yoghurt preparation and pH changes

Yoghurt was prepared according to the procedure described in Section 3.2.3.

8.2.4 Determination of cell growth during storage

Cell population of *L. delbrueckii* ssp. *bulgaricus* Lb1466, *S. thermophilus* St1342, *L. acidophilus* L10, *L. casei* L26 and *B. lactis* B94 were determined according to the procedure described in Section 3.2.4.

8.2.5. Determination of proteolytic activity by SDS-polyacrylamide gel electrophoresis

The extent of proteolysis in the control and probiotic yoghurts were monitored by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using the stacking gel system as described by Laemmli (1970) and Ong *et al*. (2006). Briefly an aliquot of each yoghurt sample (0.26g) was added to a mixture of 1.5 mL of 10 mM Tris (Sigma) and 1 mM EDTA pH 8.0 buffer, 350 µL of 10% SDS (Sigma) and 50 µL of β-mercarptoethanol (Bio-Rad Laboratories Ltd., Watford, UK). The samples were boiled in a water bath for 10 min till the mixture was clear. An aliquot of 25 µL of the sample stock solution was diluted with 100 µL of 2 x treatment buffer solution (0.13 M Tris-HCl, 4% SDS, 20% v/v glycerol, 0.2 M dithiothreitol, 0.02% bromophenol blue) (Sigma). The pH of the treatment buffer solution was adjusted to pH 6.8. A 2 mg/mL each of α-, β- casein (Sigma) were prepared in deionised water at pH 7.0 and 20 µL of each solution was diluted with 40 µL buffer solution. The

yoghurt samples, α- and β- casein were each loaded (15 µL) into 12.5% running gels. A 20 µL broad rang pre-stained SDS-PAGE standards (Bio-Rad Laboratories, Hercules, CA USA) was used as a marker. SDS-PAGE gels were run in a bio-Rad Protean II xi cell filled with buffer solution made of 0.03 M Tris, 0.19 M glycine, 0.1% SDS, adjusted to pH 8.3, and fitted with a power pac 300 set at 50mA. The gels were fixed in de-staining solution I made up of 40% methanol and 7% acetic acid for 30 min and then stained in a staining solution of 0.03% Coomassie Brilliant blue (ICN Biochemicals Inc., Aurora, OH, USA), 40% methanol and 7% acetic acid for 4h. The gels were then de-staining in de-staining solution I for 1h followed by de-staining solution II (7% acetic acid, 5% methanol) until the background became clear. The gel images were recorded using a Fuji Film Intelligent Dark Box II with Fuji Film Las-1000 Lite V1.3 software. The area and intensity of the bands were measured using Fuji Film Image Gauge V4.0 software (Fuji Photo Film Co. Ltd., Japan). Data obtained were expressed as the ratio of the area and the intensity of the band. The reduction in the intensity of bands during storage with respect to the original intensity was expressed as percentage of hydrolysis.

8.2.6 Proteolytic activity by OPA method

The degree of proteolysis during fermentation of milk was further confirmed according to the procedure described in Section 3.2.6.

8.2.7 Preparation of water-soluble peptides extract

Yoghurt samples and heat treated RSM, 250 mL of each with pH adjusted to 4.55 at days 1, 7, 14, 21 and 28 of storage, were centrifuged at 15,000 x g (J2-HS rotor, Beckman Instruments Inc., Palo Alto, CA, USA) at 4^{o}C for 30 min. The supernatant was filtered through a 0.45 µm membrane filter (Schleicher & Schuell GmbH, Dassel, Germany) and the filtrate was freeze dried (Dynavac freeze drier; Dynavac Eng. Pty. Ltd., Melbourne, Australia) and stored at -80^{o}C for further analysis.

8.2.8 Determination of ACE-inhibitory activity

The ACE-inhibitory activity was measured according to the procedure described in Section 4.2.6.

8.2.9 RP-HPLC analysis of water-soluble peptides extract

The water soluble peptides in the control and probiotic yoghurts and the RSM samples were profiled on a reversed-phase HPLC (Varian Analytical Instruments, Walnut Creek, CA, USA) equipped with C-18 monomeric column (5 μm, 300 Å, 250 mm x 4.6 mm; Grace Vydac, Hesperia CA, USA). Samples were applied using a 100 μL injection loop. The peptides were eluted by a linear gradient from 100 - 0% solvent A (0.1% trifluoroacetic acid (TFA) in deionised water) in solvent B (0.1% TFA in 90% v/v acetonitrile in deionised water) over 90 min. Separations were conducted at room temperature (~20^{o}C) at a flow rate of 0.75 mL min^{-1}. The eluted peptides were detected at 214 nm using a Varian 9050 variable wavelength UV/vis detector. All samples and solvents were filtered through a 0.45 μm membrane filter (Schleicher & Schuell GmbH).

8.2.10 Isolation, purification and analysis of peptides from yoghurt extract

The freeze dried water-soluble extract (2.5 g) of probiotic and control yoghurts was dissolved in 5 mL aliquots of 0.1% TFA in deionised water, and centrifuged at 14,000 x g with Eppendorf 5415C centrifuge (Crown Scientific, Melbourne, Australia) for 30 min. The supernatants were filtered through a 0.45 μm membrane filter into HPLC vials for peptide analysis. Fractions from five successive RP-HPLC runs under conditions described above were collected manually at 1 min intervals, evaporated in a freeze drier, redissolved in 60 μL deionised water and assayed for ACE-I activities as described in section 2.7. The ACE inhibitory activities and IC_{50} values of probiotic and control yoghurts as well as the formation degradation kinetics of specific ACE-I peptides of probiotic yoghurt were determined during storage.

Probiotic yoghurt after 28 days of storage was selected for isolation and characterization of specific ACE-I peptides by amino acid sequence analysis. Fractions were collected and treated as described above and the most potent inhibitory activity fractions were subjected to further purification analysis. Twenty-one fractions showed ACE-inhibitory activity and were further subjected to purification processes by multiple-step reversed phase chromatography. After inhibitory test at each purification step, 8 fractions exhibited high ACE-inhibitory activity and were labelled as peak 1 – 8, according to the elution order with concentrations ranging from (1.56 – 12.41) μg/mL and the purity of the fractions was confirmed by a capillary electrophoresis (CE) analysis before characterization by N-terminal amino acid sequencing by automated Edman degradation chemistry.

Capillary electrophoresis system consisted of a coated capillary (50 cm x 50 μm), a Beckman P/ACE system 5010 and a Beckman P/ACE Station software version 1.0 (Beckman Instruments Inc., Fullerton, CA, USA). The separation was performed using 30 mM sodium borate and 17 mM sodium phosphate buffer pH 8.2 at 15 kV for 30 min. Peaks were detected at 214 nm and the separation (capillary) temperature was maintained at 20^{o}C. The amino acid sequence of purified peptides was determined using automated Edman degradation on Applied Biosystem Procise protein/peptide sequencer (Applied Biosystem, Uppsala, Sweden).

8.2.11 Statistical analysis

The experiments were organized as a random, full factorial design exploring the influence of probiotic cultures and time as the main effect. All experiments were replicated and sub-sampled at least twice (n = 6). Results were analyzed as a split plot in time design using General Linear Model (GLM) procedure of the SAS System (SAS, 1996). The univariate ANOVA test was validated by fulfilling Huynh-Feldt (H-F) condition (Littell *et al*., 1998). Linear regression and correlational analysis were employed using Microsoft® Excel StatProTM (Albright *et al*., 1999) and the multicomparison of means was assessed by Tukey's test. The level of significance was preset at $p \leq 0.05$.

8.3 RESULTS

8.3.1 pH change and cell growth during storage

During yoghurt manufacture, the pH of RSM declined from 6.50 to 4.50 for both batches when the gel was formed and fermentation was terminated at an incubation time of 3.50 and 4.55 h for probiotic and control yoghurts, respectively. The pH of control yoghurt increased from 4.50 to 4.55 at 12 h of storage at 4^{o}C and then declined slightly during cold storage, effect commonly known as 'post acidification'. A similar trend was observed for probiotic yoghurt. The decline in pH after 12 h storage for both batches ($P < 0.05$) varied between 4.45 to 4.21 for the probiotic yoghurt and 4.55 to 4.35 for the control yoghurt at the end of storage as shown in Figure 8.1.

Cell growth of *L. delbrueckii* ssp. *bulgaricus* Lb1466, *S. thermophilus* St1342, *L. acidophilus* L10, *L. casei* L26 and *B. lactis* B94 upon inoculation, after fermentation and during storage at 4^{o}C is shown in Figure 8.2. *S. thermophilus* St 1342 grew well (> 8 log cfu/g) and showed consistent increase in cell concentration in both control and probiotic yoghurts during storage, whereas *L. delbrueckii* ssp. *bulgaricus* Lb 1466 exhibited a decline

($P < 0.05$) in growth (7.85 to 6.93 log cfu/g) in control yoghurt. However, the growth of *L. delbrueckii* ssp. *bulgaricus* Lb 1466 in probiotic yoghurt improved ($P < 0.05$) compared to the control yoghurt. The survival of the probiotic organisms was not significantly ($P > 0.05$) different among the organisms during storage with the exception of *B. lactis* B94 which declined ($P < 0.05$) in cell counts at the end of storage (Figure 8.2).

8.3.2 Proteolytic activity

The electrophoretic pattern of SDS-PAGE of yoghurt during storage at 4^{o}C showing the extent of proteolysis is presented in Figure 8.3. The patterns obtained allowed the identification of the different casein fractions. Protein bands were identified based on comparison with molecular weight of pre-stained SDS-PAGE standards (Bio-Rad) and α- and β- casein standards (Sigma). SDS-PAGE pattern was different between control yoghurt and probiotic yoghurt. This indicates that the proteolytic profile of the control yoghurt may differ from the probiotic yoghurt. The concentration of lower molecular weight breakdown products of the caseins appeared to be increasing with storage time as shown in the bands below β- casein (Figure 8.3). On the other hand, the concentration of casein decreased due to the proteolytic activities of the starter cultures. Ong *et al*, 2006, reported similar findings using SDS-PAGE to monitor the ripening of Cheddar cheese. Thus Rank *et al*. (1985) defined primary proteolysis as those changes α_s-, β-, and γ-caseins and peptides, which can be detected by PAGE.

Proteolytic activity of *L. delbrueckii* ssp. *bulgaricus* Lb1466, *S. thermophilus* St1342 and probiotic bacteria (*L. acidophilus*, *L. casei and B. lactis*) in yoghurts assessed during manufacture and storage using the OPA method is shown in Figure 8.4. In general, appreciable proteolytic activity was detected in both cases. The free amino acid content in both yoghurts was higher due to proteolytic activity of microorganisms compared to RSM as control. Proteolysis, as assessed by the release of free NH_3 groups by OPA method, increased ($P < 0.05$) significantly during the first 24 h in storage after manufacture and continued to increase ($P > 0.05$) at a slower rate during storage for both yoghurts (Figure 8.4). However, proteolysis in the probiotic yoghurt was significantly ($P < 0.05$) higher (~ 35%) than that of the control yoghurt after fermentation, and the level of proteolytic activity remained substantially higher (~ 20%) than the control yoghurt throughout storage at 4^{o}C.

8.3.3 Water–soluble peptides of yoghurts in comparison to RSM

Figures 8.5 A and B show RP-HPLC peptide profile of yoghurts during storage and that in RSM as separated using RP-HPLC. Three samples each of water soluble peptides of probiotic yoghurt, control yoghurt and RSM were subjected to RP-HPLC analysis. The chromatograms of the two yoghurts during storage showed substantially higher levels of liberated peptides compared to that of RSM stored under identical conditions but with no added culture. The peptide pattern of probiotic yoghurt also substantially differed from that of the control which indicated differences in the proteolytic patterns of the two types of yoghurt during storage at 4^{o}C. Comparison of chromatograms in Figures 8.5A and B showed that water soluble peptides in control yoghurt eluted predominantly in the intermediate/high hydrophobic region of the chromatogram, however, probiotic yoghurt contained more peptides eluting in hydrophilic region or within the first 20 min of RP-HPLC separation. This observation suggested higher peptidase activity in the probiotic yoghurt.

8.3.4 ACE-inhibitory activities and IC_{50} of probiotic yoghurt and control samples

Figure 8.6 presents the *in vitro* ACE inhibitory activity and IC_{50} of water-soluble peptide fractions from the control and probiotic yoghurts at weekly intervals during storage at 4^{o}C. ACE inhibitory activity in the control yoghurt increased from 70 to 90% over 28 day refrigerated storage period. The IC_{50} value of the inhibitory peptide in control yoghurt during the storage period followed a downward trend after initially increasing during the first week. In contrast, water-soluble peptides from probiotic yoghurt at day 1 completely inhibited ACE and then followed a general trend of reduction in inhibitory activity. The IC_{50} values for the probiotic yoghurt peptides showed that extremely potent ACE inhibitory peptides were produced at day 1 but during the first week in the storage period potent ACE inhibitors appeared to be further hydrolysed as seen from the sharp increase in IC_{50} at day 7. However, the IC_{50} values decreased ($P < 0.05$) sharply between day 7 and 21 indicating further proteolytic modification of inactive peptide or release of new peptides from caseins. In the last week of storage, ACE inhibition of peptides in probiotic yoghurt declined significantly which was also reflected by the sharp increase in the IC_{50} values between day 21 and 28. No ACE inhibitory activity was detected for RSM. Nonetheless, it is evident that control yoghurt without any probiotics also showed *in vitro* ACE-I activity but the IC_{50} values were substantially higher compared to those of the probiotic yoghurts.

8.3.5 Formation / degradation pattern of specific ACE-I peptides of probiotic yoghurt during storage

Figure 8.7 shows time dependent purified water-soluble peptide fractions from probiotic yoghurt during cold storage. All the fractions of interest increased in peptide concentration from day 1 to week 3 and reached maximum concentrations. There was a slight ($P > 0.05$) decrease in fraction 8 compared to other fractions. Fractions 2 and 6 appeared constant whereas 3 and 7 continued to increase in concentration at the end of storage.

8.3.6 Purification and characterization of water-soluble peptides of probiotic yoghurt

The peptide profile was investigated for d 28 samples to establish the sequence of peptide by the end of the storage period for probiotic yoghurt. Eight most potent ACE inhibitory fractions from the RP-HPLC chromatogram of day 28 probiotic yoghurt were purified (fractions 1-8, see Figure 8.5B). Fractions were further chromatographed and collected from RP-HPLC (e. g. Figure 8.8). Purity of collected fractions was checked by CE (see Figure 8.9) before N-terminal amino acid sequencing. Table 8.1 shows the ACE inhibitory activities, IC_{50} and sequence information of the purified fractions. The eight peptides isolated from α, β and κ–casein showed potent inhibitory activities of IC_{50} values ranging from 1.56 to 12.41 μg/mL.

8.4 DISCUSSION

The high ($P < 0.05$) growth of *S. thermophilus* and their survival in both batches of yoghurt during production and storage has also been reported in various studies (Hamann and Marth, 1984; Rohm *et al*., 1990; Akalin *et al*., 2004). On the other hand, *L. delbrueckii* ssp. *bulgaricus* Lb 1466 only showed enhanced growth in the presence of probiotic organisms (Figure 8.2). The poor growth of *L. delbrueckii* ssp. *bulgaricus* Lb1466 in control yoghurt was likely affected by the decline in pH ($r = 0.81$). Similar observations of poor growth of *L. delbrueckii* ssp. *bulgaricus* in control yoghurt during cold storage was reported in Section 3.4. However, *S. thermophilus*, *L. acidophilus* L10 and *L. casei* L26 were not affected by the decline in pH in yoghurt throughout the storage (Figure 8.2). These organisms have been observed in Section 3.4 to tolerate acidic conditions better than *Bifidobacterium* sp. The decline in the population of *Bifidobacterium* sp. observed in our study in yoghurt during cold storage confirmed a previous report (Dave and Shah, 1997),

which showed a poor survival of *Bifidobacterium* sp. after yoghurt manufacturing. The changes in pH could have been the most crucial factor for the survival of *B. lactis* B94 since the decline from day 21 to 28 was substantial and might have resulted in a decrease ($P < 0.05$) in cell counts ($r = 0.52$) for these microorganisms compared to other probiotic bacteria (Shah and Lankaputhra, 2002; Akalin *et al*., 2004). Several other factors have been claimed to affect the viability of probiotic bacteria in yoghurt including acidity, hydrogen peroxide, oxygen content, concentrations of lactic acids, and temperature of storage during manufacture and storage of yoghurt (Lankaputhra and Shah, 1995; Samona and Robinson, 1994; Dave and Shah, 1997; Shah, 2000b; Shah and Ravula, 2000; Talwalkar and Kailasapathy, 2004). This indicates that some organisms competed better and remained viable in yoghurt than others up to 28 d of storage at 4^{o}C.

Generally probiotic microorganisms achieved substantial cell growth above the therapeutic minimum of 6.00 log cfu/g, mostly attaining maximum cell counts up to day 7 and 14 (Figure 8.2). The fact that high populations of probiotic organisms were detected in this study showed that these bacteria were not antagonistic towards each other in yoghurts as have been reported in the literature (Dave and Shah, 1997). Their coexistence may be mutually beneficial as in case of reported non-antagonistic relationship between *L. acidophilus* and *Bifidobacterium* sp. in fermented milk (Klaver *et al*., 1990). On the other hand, the decline in cell counts for some microorganisms may also be due to a nutrient depletion in the growth media (Gilliland and Speck, 1977). The growth most likely was sustained by the proteolytic activity as illustrated by a positive correlation ($r = 0.36$ to 0.66) for *L. delbrueckii* ssp. *bulgaricus* Lb1466, *S. thermophilus* St1342, *L. acidophilus* L10 and *L. casei* L26. However, the extent of proteolysis had no apparent effect on the growth of *B. lactis* B94 ($r = -0.78$) in probiotic yoghurt during storage, which was similar to a previous report (Schieber and Bruckner, 2000).

Although yoghurt culture exhibited a substantial level of proteolytic activity in the control yoghurt, the amount of liberated peptides was further enhanced by the action of probiotic bacteria in the probiotic yoghurt (Figure. 8.3 and 8.4). This may suggest high demand for essential growth factors such as peptides and amino acids by the probiotic organisms during their growth in milk. Consequently, the increased proteolytic activity could have resulted in better survival of some probiotic organisms in yoghurt during storage as reported in Section 3.4.

The extent of released peptides increased as expected during cold storage of yoghurts. This is illustratively presented by the HPLC elution profile of the peptides

obtained from the water soluble fractions (Figures. 8.5 A, B). Interestingly, the pattern of the time-dependent peptide profile was different for probiotic yoghurt compared to that prepared with yoghurt culture alone. This may indicate differences in proteinase and/or peptidase specificity in the various microorganisms. Although the peptide profiles differed, we found in a preliminary study that both yoghurt batches inhibited ACE activity significantly at different levels in comparison to RSM. Gobbetti *et al*. (2000) and Fitzgerald and Murray (2006) also reported on various peptides isolated from yoghurt and milk proteins that showed inhibitory activity towards ACE. Apparently the types and quality of peptides produced in the two batches of yoghurt in our study appeared to be different as shown by the IC_{50} values (d 1 to d 28) of the yoghurt made with *S. thermophilus* St1342 and *L. delbrueckii* ssp. *bulgaricus* Lb1466 alone and probiotic yoghurt. 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). Thus Calpis® sour milk, which is fermented with *Lactobacillus helveticus* and *Saccharomyces cerevisiae*, had been the source of two potent ACE-I peptides (Nakamura *et al*., 1995).

The rate of hydrolyses/degradation of the bioactive peptides by the cultures during 3 to 4 weeks of storage likely depended on type of peptide (amino acid sequence) since some were hydrolysed faster than the others (Figure 8.7). The end results may indicate that some peptides of interest may disappear and new ones may appear during prolonged storage (Gobbetti *et al*., 2000). On the other hand, peptides 2, 3, 6, and 7 resisted hydrolysis by the cultures and their concentrations either increased or remained constant during fermentation and storage, reaching maximum concentrations at 3.77, 6.09, 2.61, and 1.56 µg/mL, respectively, in the product. Korhonen and Pihlanto (2006) reported a similar finding of increased concentration of an ACE-I peptide from a yoghurt-like product fermented with *Lb. helveticus* strain. Two of the isolated peptides, namely VPP and IPP, are well-known ACE-inhibiting peptides used in commercial products. Their concentrations appeared within a range reported in the literature (Fitzgerald and Meisel, 2000). Therefore, the probiotic yoghurts described here may be of commercial interest, in addition to the products already available on market including Calpis® sour milk (Calpis Co. Ltd., Tokyo, Japan) and Evolus® brand (Valio Ltd., Finland), respectively (Sipola *et al*., 2002; Hata *et al*., 1996).

Most of the documented ACE-I peptides are usually short peptides with a proline residue at the carboxyl terminal end. Proline is known to be resistant to degradation by digestive enzymes and may pass from the small intestine into the blood circulation in the sequence of short peptides (Korhonen and Pihlanto, 2006; Pan *et al*., 2005). However, most

of dairy cultures possess X-prolyl-dipeptidyl aminopeptidase capable of cleaving tri- and oligopeptides with proline in the penultimate position, which subsequently may result in a disappearance of potent ACE-I such as VPP or IPP (Section 6.3.1). Fraction 7 is of particular interest as it showed the most potent *in vitro* ACE-I peptide (Table 8.1). Further tests on this peptide are necessary which may reveal a new peptide, and thus enhance the functionality of probiotic yoghurt.

Fractions of interest (numbered 1- 8) of day 28 yoghurt gave single peaks after further purification with HPLC (fraction 4, Figure 8.8). These fractions were finally subjected to CE analysis, which confirmed their purities (fraction 4, Figure 8.9) and their structural analysis provided the data summarized in Table 8.1. By the initial sequence analysis, the two peptides identified were the tripeptides Val-Pro-Pro (VPP; peak 6) and Ile-Pro-Pro (IPP; peak 2). VPP and IPP showed higher *in vitro* ACE-I activity than most of the other inhibitory peptides identified in this study and similar to other reported ACE-I peptides purified from milk products (Yamamoto *et al*., 1994a, b; Maeno *et al*., 1996; Pihlanto-Leppalla *et al*., 1998; Takano, 1998; Fitzgerald and Meisel, 2000; Gobbetti *et al*., 2002; Leclerc *et al*., 2002; Seppo *et al*., 2003). The IC_{50} values of the peptides were 2.61 ± 0.24 and 3.77 ± 0.26 μg/mL or (8.4 and 11.6 μM) respectively. VPP and IPP are obtained from hydrolysed skim milk with *Lb. helveticus* and *S. cerevisiae*, showing IC_{50} values at 9.13 ± 0.21 and 5.15 ± 0.17 μM, or 2.8 and 1.6 μg/mL respectively (Nakamura *et al*., 1995; Fitzgerald and Meisel, 2000; Pan *et al*., 2005). Amino acid sequences of VPP and IPP peptides are found in the primary structure of bovine β-casein (CN)-f84-86 and β-CN-f74-76 or κ-CN-108-110, respectively (Yamamoto *et al*., 1994b; Pan *et al*., 2005). These amino acid sequences were cleaved by proteinase, with further modification by peptidases, from the starter cultures during fermentation of RSM (Kunji *et al*., 1996; Christensen *et al*., 1999; Pan *et al*., 2005). Five N-amino acids determined for peptide identified in peak 3 were Tyr-Gln-Glu-Pro-Val-Leu, which perfectly matched with the primary structure of bovine β-CN-f193-198 (Schieber and Bruckner, 2000). The amino acid sequence data made it possible to locate the five N-terminal residues on the β-CN structure but the C-terminal end of the peptide may or may not be the residue Leu^{198} due to physico-chemical limitation of amino acid sequencing chemistry. In order to conclusively state C-terminal end or the exact length of the peptide on the β-CN primary structure will require molecular weight determination by MS. In our study, β-CN-f193-198 was detected throughout storage of yoghurt and we therefore assumed that it accumulated in the product with an IC_{50} value at 6.09 ± 0.46

μg/mL (Table 8.1). Several peptides from β-CN originating from the region 193-198, including the parent large fragment, has been reported earlier to exhibit ACE-I activity (Pihlanto-Leppalla *et al*., 1998). Peptide β-CN-f193-209 has been previously identified in several cheese varieties (Singh *et al*., 2003) and yoghurt (Schieber and Bruckner, 2000). In several cheese varieties, peptide β-CN-f193-209 was associated with the bitter taste defect (Singh *et al*., 2005). This peptide in cheese can be produced by the action of rennet or cell wall associated proteinase of starter bacteria on peptide bond Leu^{192}-Tyr^{193} of β-CN (Singh *et al*., 2005). In the present study, presence of peptide β-CN f193-198 in probiotic yoghurt clearly showed the ability of bacterial proteinase to hydrolyse peptide bond Leu^{192}-Tyr^{193}. In a recent study, Miguel *et al*. (2006) showed antihypertensive activity in SHR rat model of peptides β-CN f197-206 and f201-209, identified in *Enterococcus faecalis*-fermented milk.

Three of five ACE-I β-CN peptides in the present study originated from the region 50-106 of β-CN. In excess of one hundred peptides were identified from Cheddar cheese originating from region 50-106 (Singh *et al*., 1995, 1997) and once formed were found to accumulate over the period of ripening. Several peptides were identified in yoghurt by Schieber and Bruckner (2000) and 26 peptides out of a total of 30 were found to originate from two distinct regions of β-CN, i.e. 30-75 and 120-209. Quite diverse range of biological activities were found to originate from the region 50-80 of β-CN, e.g. ACE inhibition, opioid activity, starter bacterial peptidase inhibitor (Gobbetti *et al*., 2002). In addition to ACE-I β-CN peptides, two ACE-I peptides from κ-CN and one from α_{s2}-CN were also identified in the probiotic yoghurt samples. Little or no information is available on the proteinase/peptidase profiles of the probiotic microorganisms. In addition to enzyme profile, data on specificity of proteinases and peptidases will be useful in the production of specific biologically active peptide(s) and in regulating their levels.

8.5 CONCLUSIONS

The pattern of time-dependent peptide profile may indicate differences in proteinase specificity in the various microorganisms in probiotic and control yoghurts. Yoghurt produced by probiotic bacteria as adjunct culture exerted appreciable *in vitro* ACE-I activity. The enhanced proteolytic activity likely contributed to the observed inhibition. Several potential *in vitro* ACE-I peptides were identified from casein and these were found to be active inhibitors. However, further studies are necessary to elucidate the formation and degradation mechanism of these peptides. The development of yoghurt containing higher

concentrations of released bioactive ACE inhibitors and viable probiotics may deliver health benefits to consumers.

Table 8.1 The angiotensin I-converting enzyme (ACE) inhibitory activity of purified peptides from yoghurt

Peak	Sequence*	Origin**	% ACE-I$^{\varphi}$	$IC_{50}^{\#}$ (μg/mL)
1	Thr-Tyr-Lys-Glu-Glu	α $_{52}$CN-f19-23	52.35 ± 0.84	12.41± 0.46
2	Ile-Pro-Pro	β-CN-f74-76	68.60 ± 0.10	3.77± 0.26 (11.6 μM)
		κ-CN-108-110		
3	Tyr-Gln-Glu-Pro-Val-Leu	β-CN-f193-198	48.60 ± 2.03	6.09 ± 0.46
4	Arg-Ile-Asn-Lys-Lys	β-CN-f25-29	44.80 ± 0.31	12.05 ± 0.93
5	Ser-Leu-Pro-Gln-Asn	β-CN-f69-73	43.43 ± 2.04	5.29 ± 0.55
6	Val-Pro-Pro	β-CN(f84-86)	57.41 ± 0.48	2.61 ± 0.24 (8.4 μM)
7	ND$^{\sigma}$	ND$^{\sigma}$	75.42 ± 1.34	1.56 ± 0.27
8	Ala-Arg-His-Pro-His	κ-CN-f96-100	39.83 ± 2.27	9.64 ± 3.67

*N-terminal amino acid sequence determined by Edman degradation method.
**Origin and the position in each casein molecule are indicated
$^{\#}$The concentration of peptide needed to inhibit 50% of the ACE activity
$^{\varphi}$Mean of six determinations
$^{\sigma}$ No meaningful amino acid sequence data was obtained

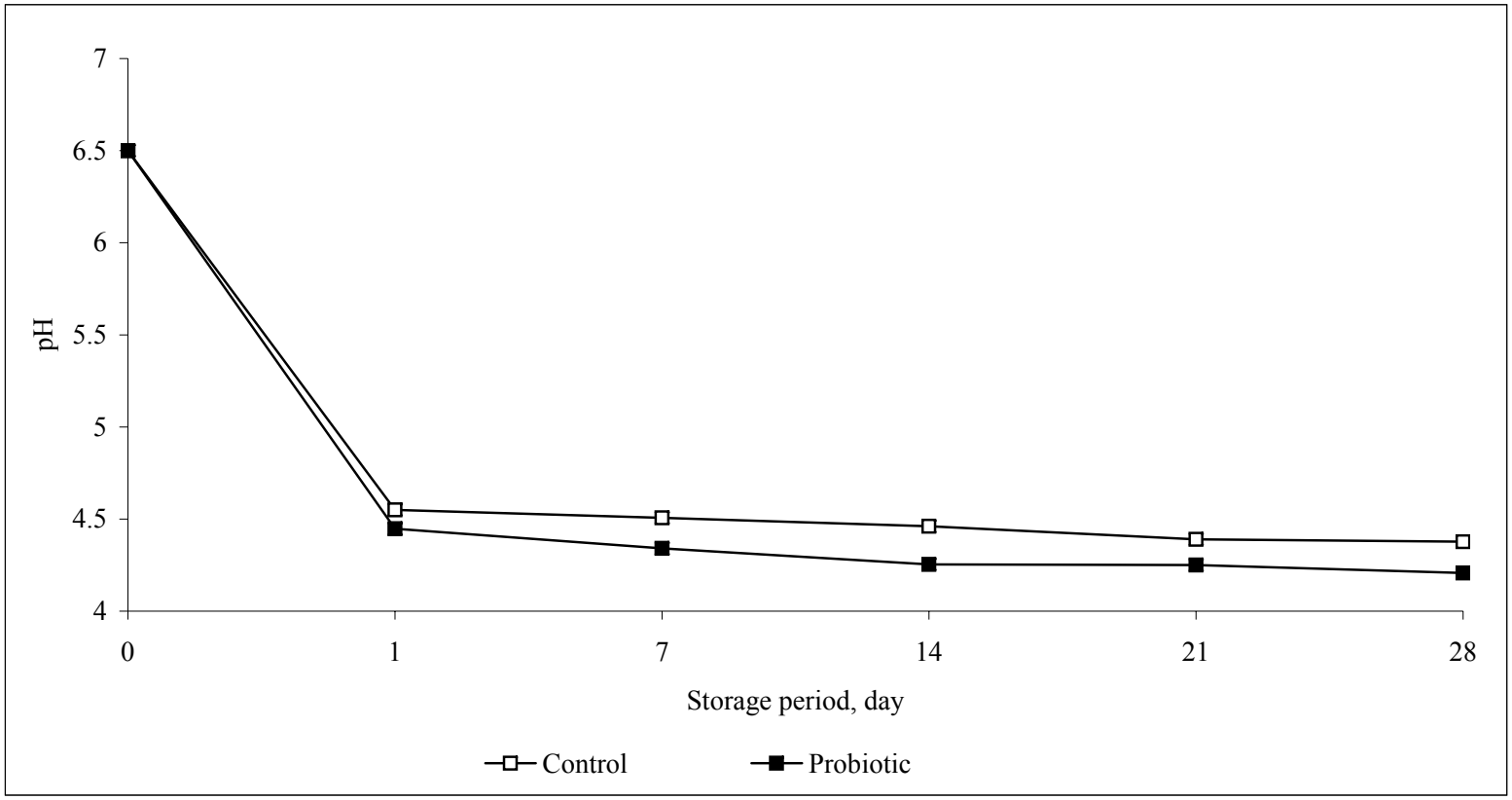

Figure 8.1 pH changes after fermentation of control yoghurt containing *S. thermophilus* St1342, *L. delbrueckii ssp. bulgaricus* Lb 1466 and probiotic yoghurt (*S. thermophilus* St1342, *L. delbrueckii ssp. bulgaricus* Lb 1466, *L. acidophilus* L10, *L. casei* L26 and *B. lactis* B94) during storage at 4^{o}C. (Error bars present a pooled standard error of the mean).

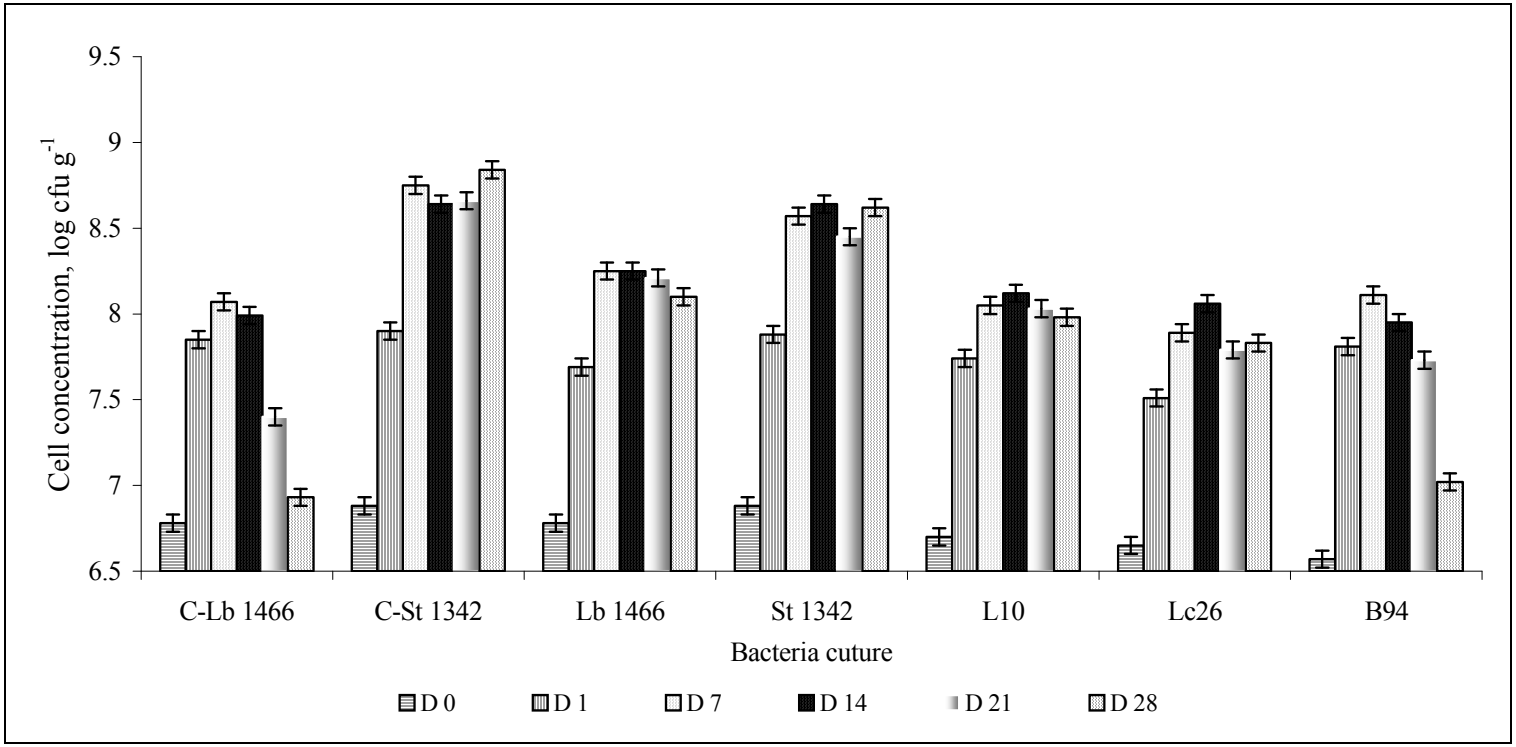

Figure 8.2 The change in cell concentration of control (C) yoghurt, which contain yoghurt culture (*L. delbrueckii* ssp. *bulgaricus* Lb1466, and *S. thermophilus* St1342) and probiotic yoghurt which contain yoghurt culture and probiotic organisms (*L. acidophilus* L10, *L. casei* L26 and *B. lactis* B94) during 28 d storage at 4^{o}C. (Error bars represent a pooled standard error of the mean, SEM = 0.05 cfu/g).

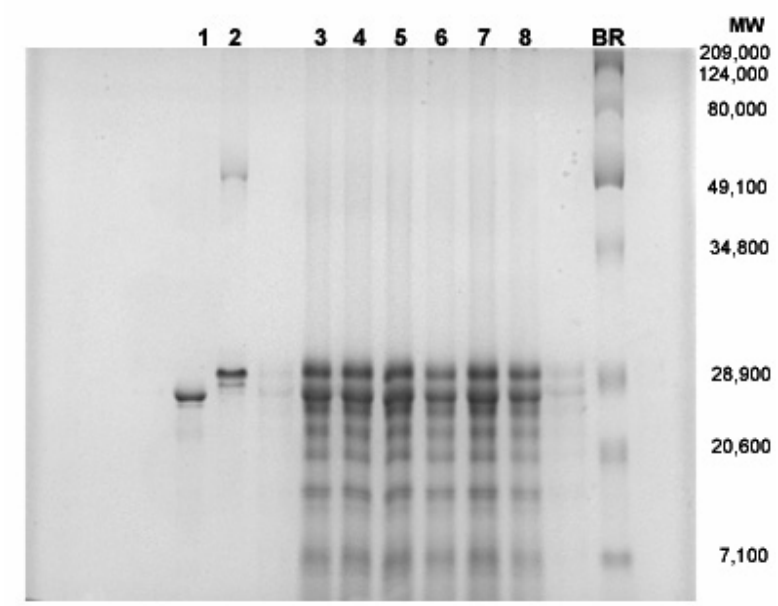

Figure 8.3 Gel electrophoretic pattern of yoghurt showing proteolytic activity during 28 D storage at 4°C. Representative SDS-PAGE of yoghurt during 4 weeks of storage at 4°C. BR=Broad rang pre-stained molecular weight (MW) standards containing: myosin (MW 205,000), β-galactosodase (MW 120,000), bovine serum albumin (MW 84,000), ovalbumin (MW 52,000), carbonic anhydrase (MW 36,000), soybean trypsin inhibitor (MW 30,2000), and lysozyme (MW 21,900); α – La = alpha-lactalbumin; β – Lg = beta-lactoglobulin; D = day; C = control.

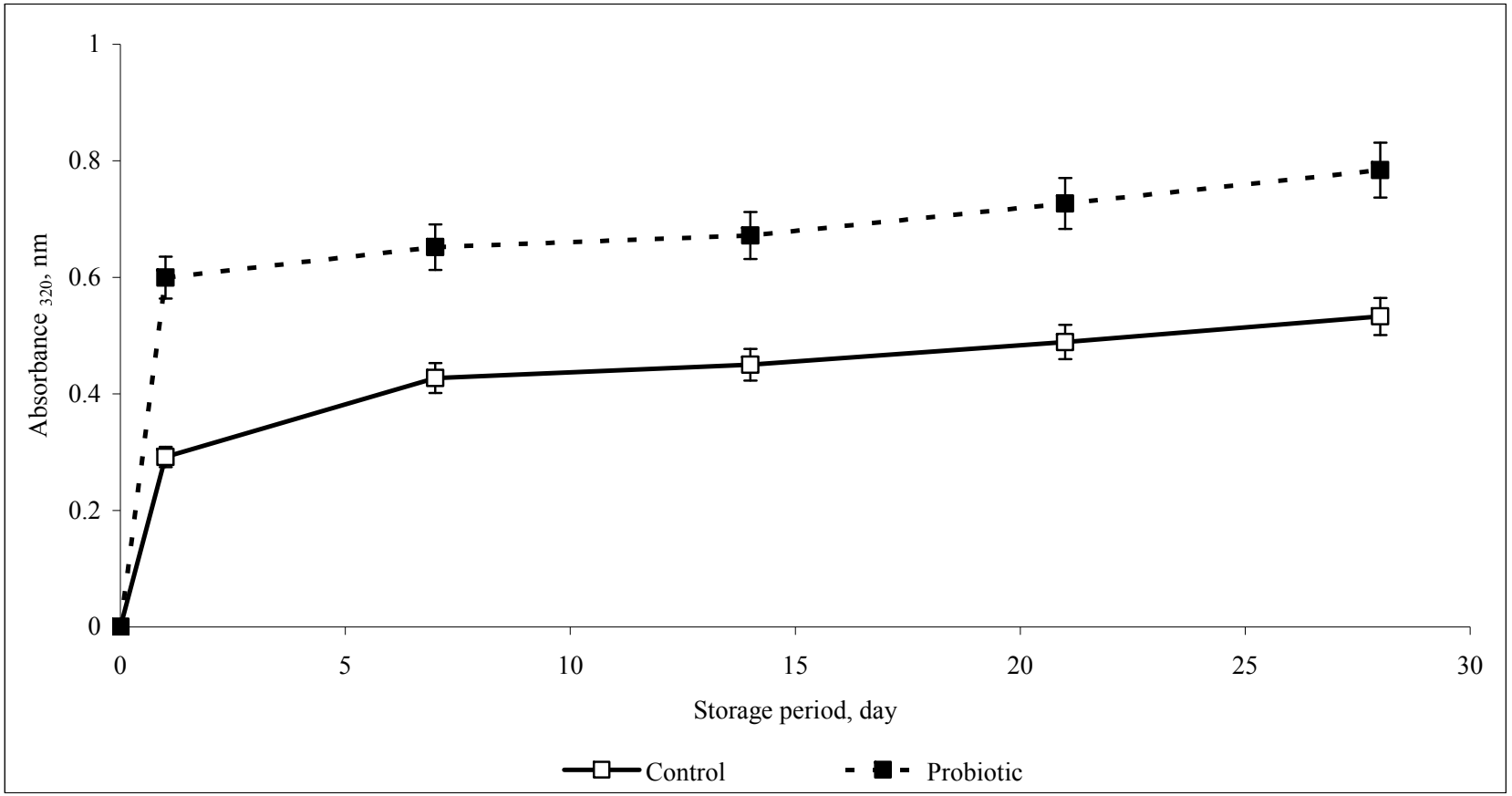

Figure 8.4 Proteolytic activity of control yoghurt, containing yoghurt culture (*L. delbrueckii* ssp. *bulgaricus* Lb1466, and *S. thermophilus* St1342), and probiotic yoghurt containing yoghurt culture and probiotic organisms (*L. acidophilus* L10, *L. casei* L26 and *B. lactis* B94) during storage at 4°C. (Error bars present a pooled standard error of the mean, 0.02).

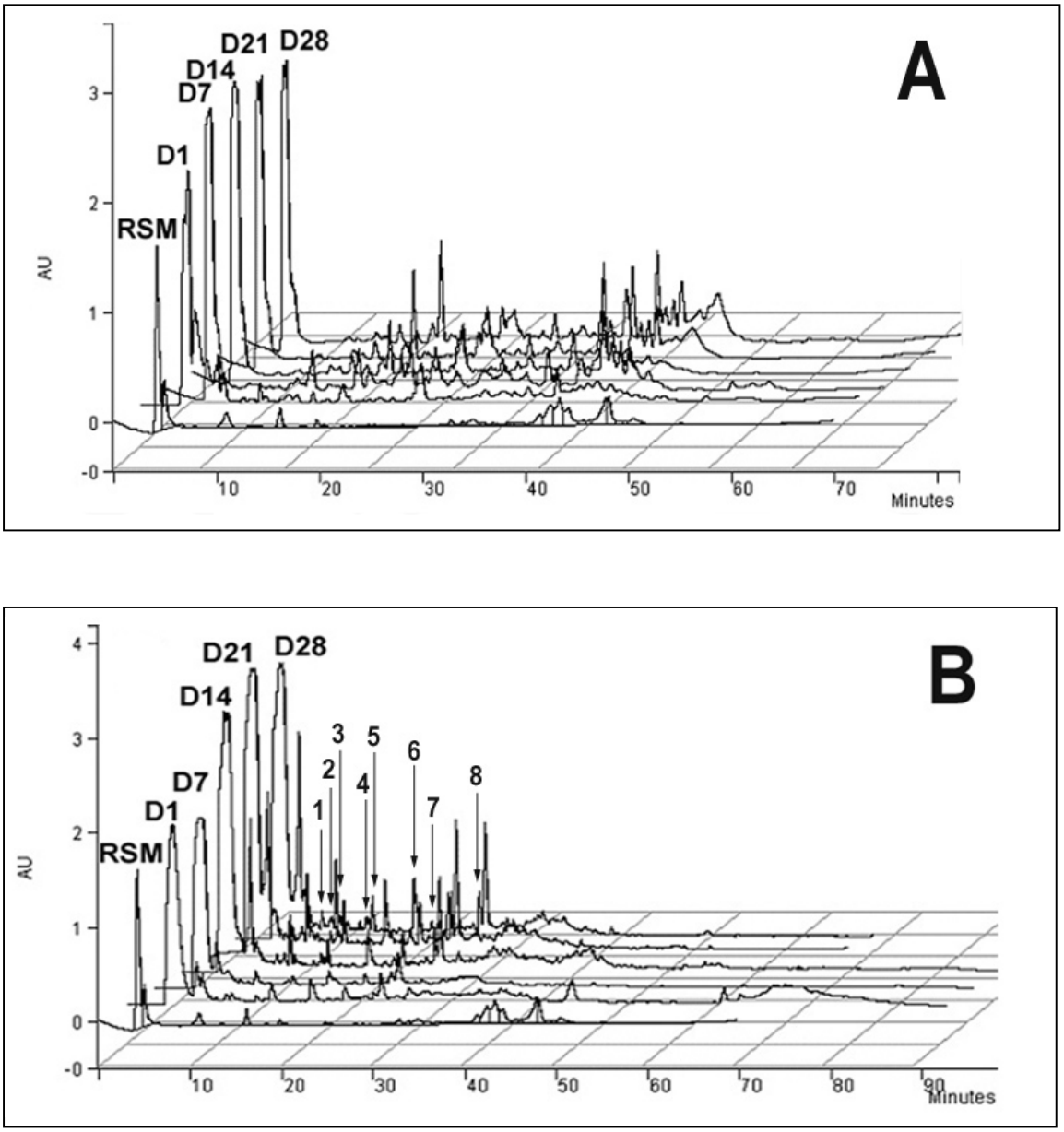

Figure 8.5 Reversed-phase HPLC profile of the water-soluble fraction of yoghurts made with yoghurt culture alone (A) and yoghurt culture and probiotic organisms (B) during 28 days storage at 4°C, compared to RSM as control during 28 days storage at 4°C. Fractions 1-8, from days 28 probiotic yoghurt sample were isolated and purified using a linear gradient from 100 - 0% solvent A (0.1% TFA in water) solvent B (0.1% TFA in 90%, v/v acetonitrile in water over 70 min at a flow rate of 0.75 mL/min. The eluted peptides were detected at 214 nm.

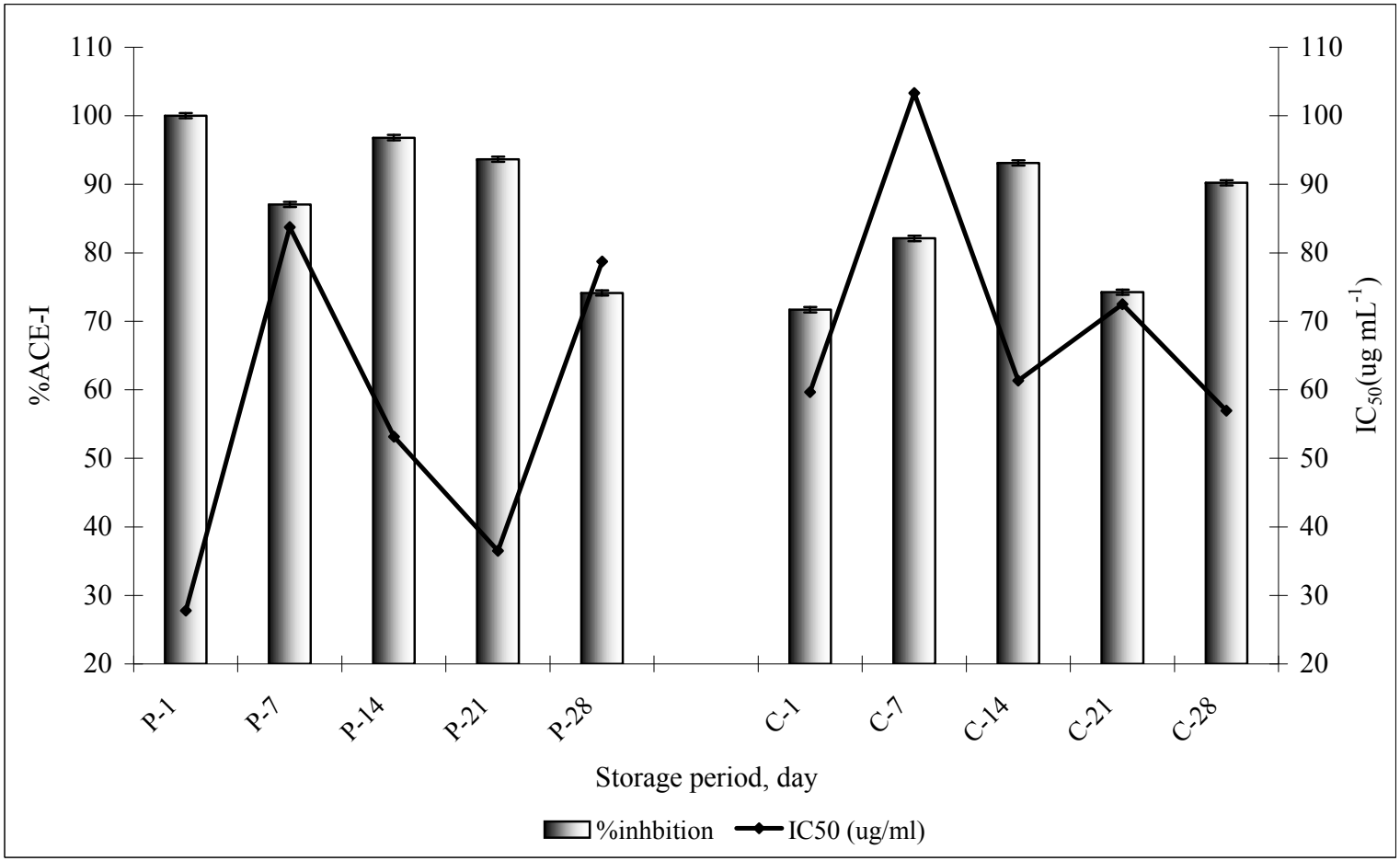

Figure 8.6 ACE inhibitory activities and IC_{50} values of probiotic and control yoghurts during 28 days storage 4°C. The extent of *in vitro* ACE inhibiting activity was calculated as a percentage. IC_{50}, was defined as the sample concentration (μg/mL) required to inhibit 50% of the ACE activity; P = Probiotic; C = Control; (Error bars present a pooled standard error of the mean).

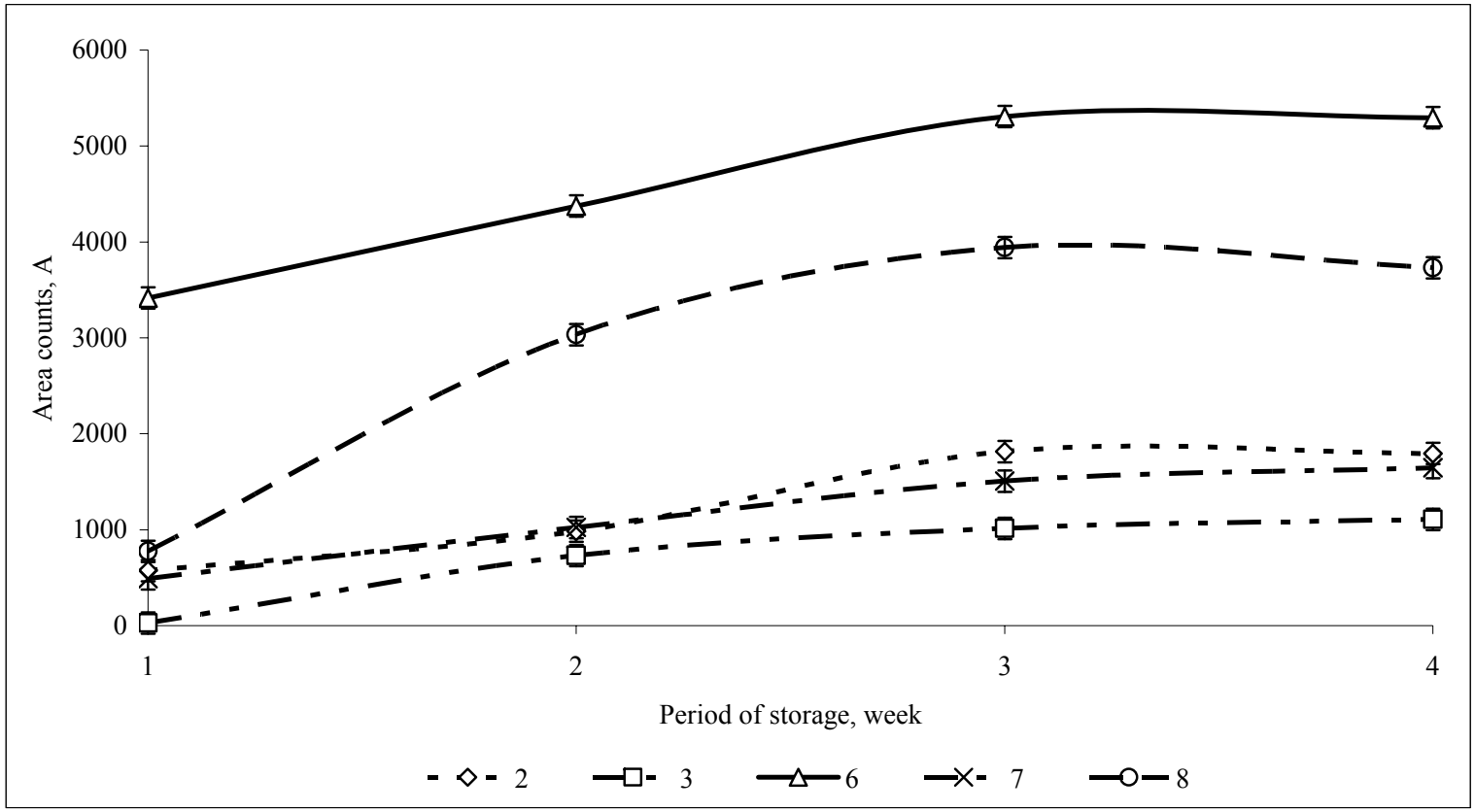

Figure 8.7 Formation degradation kinetics of ACE-I peptides showing fractions (2-8) from day 1 to week 4 of probiotic yoghurt during storage at 4°C. Monitoring of concentration (formation and/or degradation) of peptides was achieved with RP-HPLC using a C_{18} column at room temperature. The peptides were eluted by a linear gradient from 100 – 0% solvent A (0.1% TFA in water) solvent B (0.1% TFA in 90%, v/v acetonitrile in water over 70 min at a flow rate of 0.75 mL/min. The eluted peptides were detected at 214 nm. (Error bars present a pooled standard error of the mean).

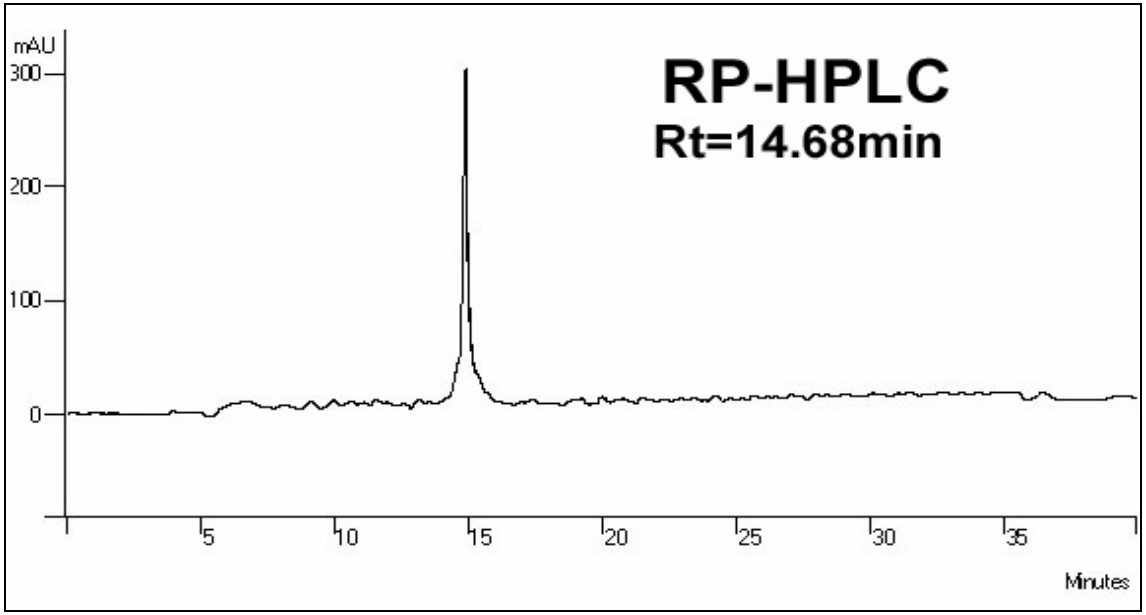

Figure 8.8 RP-HPLC chromatogram of purified fraction 4. The peptide was eluted by a linear gradient from 100 – 0% solvent A (0.1% TFA in water) solvent B (0.1% TFA in 90%, v/v acetonitrile in water over 40 min at a flow rate of 0.75 mL/min. The eluted peptide with retention time of 14.68 min was detected at 214 nm. The purity of active fraction was confirmed.

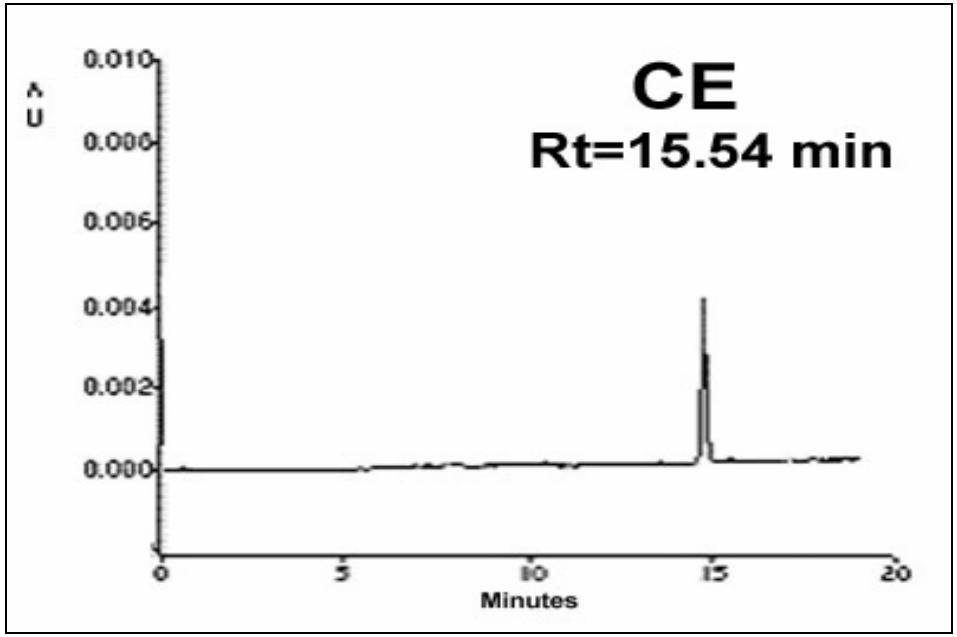

Figure 8.9 Capillary electrophoresis (CE) electrophoregram of purified fraction 4 of retention time 15.54 min. The purity of active fractions was confirmed using a coated capillary (50 cm x 50 μm), 30 mM sodium borate and 17 mM sodium phosphate buffer pH 8.2 at 15 kV for 30 min. Peaks were detected at 214 nm. The capillary temperature was maintained at 20^{o}C.

9.0 Production of β-glucosidase and hydrolysis of isoflavone phytoestrogens by *Lactobacillus acidophilus*, *Bifidobacterium lactis* and *Lactobacillus casei* in soymilk

9.1 INTRODUCTION

In addition to serving as a delivery medium for probiotic organisms to the consumer, soymilk has several nutritional advantages over dairy milk including reduced level of cholesterol and saturated fat as well as the absence of lactose. Furthermore, soy products contain the isoflavone phytoestrogens with potential anticarcinogenic activity (Favaro Trindade *et al*., 2001). Their antioxidant ability may also prevent oxidative damage in living tissue (Ruiz-Larrea *et al*., 1997; Wei *et al*., 1995). Isoflavones occur naturally in plants, in particular soybeans. Isoflavones are a part of the diphenol compounds, which are structurally and functionally similar to the human estrogen, estradiol, but are much less potent than estradiol (Setchell and Cassidy, 1999; Setchell, 1998). Because of this similarity, isoflavones were suggested to have preventive effects for many kinds of hormone-dependent diseases (Uzzan and Labuza, 2004). Isoflavones in soy proteins in most soy foods are conjugated with sugars. The β-glucoside forms are not absorbed and require hydrolysis for bioavailability and subsequent metabolism. Hydrolysis occurs along the entire length of the intestinal tract by the action of both the brush border membrane- and the bacterial β-glucosidases (Setchell *et al*., 2002). The aglycones are released and further metabolism of daidzein and genistein takes place. Intestinal biotransformations include dehydroxylation, reduction, C-ring cleavage and demethylation. These reactions take place distally and presumably in the colon. However, the types of intestinal bacteria involved in isoflavone conversion to bioactive forms and the effectiveness of this microbial biotransformation are not well known. *Bifidobacterium* and *Lactobacillus* are the predominant members of the intestinal microflora, however, *Bifidobacterium* strains are widely studied for the production of β-glucosidase (Tochikura *et al*., 1986, Tsangalis *et al*., 2002; Hsu *et al*., 2005) leading to transformation of isoflavones to bioavailable and bioactive forms (Tsangalis *et al*., 2002; Tsangalis *et al*., 2004; Wei *et al*., 2007). However, production of β-glucosidase by other groups of bacteria has not been studied. Therefore, it will be interesting to study other groups of bacteria for the production of β-glucosidase using commercial probiotic organisms (*L. acidophilus* L10, *B. lactis* B94 and *L. casei* L26) and biotransformation of isoflavone aglycones by these microorganisms in soymilk. Probiotics are defined as 'live microorganisms which when administered in adequate amounts confer a health benefit on the host' (FAO/WHO, 2002).

Donkor, O. N., & Shah, N. P. (2007). Production of β-glucosidase and hydrolysis of isoflavone phytoestrogens by *Lactobacillus acidophilus*, *Bifidobacterium lactis* and *Lactobacillus casei* in soymilk. *Journal of Food Science*, (Published on line).

The aims of this study were to evaluate β-glucosidase activity of selected probiotic organisms (*L. acidophilus* L10, *B. lactis* B94 and *L. casei* L26) for hydrolysis of isoflavone in soymilk fermented at 37°C for 48 h, and to measure the organic acids production during fermentation of soymilk at 37°C for 48 h and storage at 4°C for 28 d.

9.2 MATERIALS AND METHODS

9.2.1 Propagation of cultures

Lactobacillus acidophilus LAFTI® L10, *Bifidobacterium lactis* LAFTI® B94 and *Lactobacillus casei* LAFTI® L26 were provided by DSM Food Specialties (Moorebank, NSW, Australia). These microorganisms have been reported to have probiotic properties (Crittenden *et al*., 2005). Each culture was stored at -80°C. Sterile 10 ml aliquots of MRS were inoculated with 1% of each culture and incubated at 37°C for 20 h. For propagation of *Bifidobacterium*, sterile MRS broth was supplemented with 0.05% L-cysteine.HCl to provide anaerobic conditions and stimulate their growth (Ravula and Shah, 1998). After three successive transfers in MRS broth, the activated organisms were transferred at 5% v/v to 10 mL aliquots of sterile soymilk supplemented with 2% glucose and 1% yeast extract. After a second transfer in sterile soymilk the cultures were ready for the production of fermented soymilk.

9.2.2 Soymilk manufacture

Soymilk was prepared as per the method of Tsangalis *et al*. (2004) by mixing 4% Soy protein isolate (SPI) SUPRO® 590 IP (proximate composition: moisture 6.0%, protein, dry basis 90.0%, fat 1.5%, ash 5.0%, carbohydrate ≤ 1.0%), supplied by SOLAE Co. (Chatswood, NSW, Australia) (w/v) in deionised water. For reconstitution, deionised water was heated to 40°C prior to addition of SPI powder, followed by heating the mixture to 50°C with constant stirring for 30 min to dissolve solid particles. A 11.4 litre batch of soymilk was prepared and dispensed into nineteen glass bottles containing 300 mL each. The bottles containing soymilk were sterilised by autoclaving at 121°C for 15 min.

9.2.3 Fermentation of soymilk with micro-organisms

Two sets of six glass bottles each containing 300 mL sterile soymilk were aseptically inoculated with *L. acidophilus* L10 at 5% (v/v) and incubated at 37^{o}C for 48 h. The bottles were labeled 0, 6, 12, 24, 36, and 48 h in order to facilitate withdrawing of aliquots at 0, 6, 12, 24, 36, and 48 h of fermentation. Aliquots of 50 mL from each of the 6 bottles were taken at 0, 6, 12, 24, 36, and 48 h of incubation. Each aliquot was divided into 20 and 30 mL portions in sterile 50 mL screw top falcon tubes (Interpath Services Pty Ltd, Heidelberg Wesst, Vic., Australia). The 20 mL portions were used for determination of β-glucosidase activity, and pH, and enumeration of cell counts, whereas the 30 mL portions were immediately stored at -80^{o}C and later freeze dried using a Dynavac® FD 300 freeze drier (Rowville, Vic., Australia) for the analysis of isoflavones. The second set of 6 bottles of fermented soymilk was stored at 4^{o}C for 28 d for determination of β-glucosidase activity, pH and for enumeration of bacteria. The experiments were repeated for *B. lactis* B94 and *L. casei* L26.

9.2.4 β-Glucosidase activity in soymilk

β-Glucosidase activity of the three micro-organisms was determined in soymilk during fermentation at 37^{o}C for 6, 12, 24, 36, and 48 h. Five millilitre aliquots were taken at 0, 6, 12, 24, 36 and 48 h and the enzyme activity was determined immediately. The β-glucosidase activity was determined by measuring the rate of hydrolysis of ρ-nitrophenyl β-D-glucopyranoside (ρNPG) Sigma Chemical Company (St. Louis, MO, USA) as per the methods described previously (Scalabrini *et al*., 1998; Otieno *et al*., 2006) with some modifications. Five hundred micro-litres of 5 mM ρNPG prepared in 100 mM sodium phosphate buffer (pH 7.0) was added to 5 mL of each aliquot sample and incubated at 37^{o}C for 30 min. The reaction was stopped by the addition of 250 µL cold 0.2 M sodium carbonate (4^{o}C). The resulting mixture was centrifuged at 14,000 x g for 30 min using an Eppendorf centrifuge (model 5415C; Crown Scientific, Melbourne, Australia) and filtered through a 0.45 µm membrane filter (Schleicher & Schuell GmbH, Dassel, Germany). The amount of ρ-nitrophenol released was measured using a spectrophotometer (Pharmacia LKB® Novospec II, Uppsala, Sweden) at 420 nm. One unit of the enzyme activity is defined as the amount of β-glucosidase activity that released 1 µmol of ρ-nitrophenol from the substrate ρNPG per millilitre per min under the assay conditions.

9.2.5 Enumeration of bacterial population

Cell populations of *L. acidophilus* L10, *B. lactis* B94 and *L. casei* L26 was determined as described previously in Section 3.2.4. Briefly, one gram of each fermented soymilk sample was added to 9 mL of sterile 0.15% (w/v) bacteriological peptone (Oxoid) and water diluent and vortexed (model MT 19, Chiltern Scientific, Salmond Smith Biolab Ltd., Auckland, New Zealand) for 30 s. The resulting suspension was serially diluted in sterile 0.15% (w/v) peptone water (Oxoid) and 1 mL of the appropriate dilution was used for selective enumeration by the pour plate technique. The cell growth of each organism was assessed by enumerating bacterial population after 6, 12, 24, 36 and 48 h of fermentation of soymilk on MRS agar (Amyl media). Anaerobic jars and gas generating kits (Anaerobic system BR 38, Oxoid Ltd., Hampshire, England) were used for creating anaerobic conditions. Duplicate plates were incubated anaerobically for 72 h at 37^{o}C for *L. acidophilus, L. casei*, and *Bifidobacterium* spp. Plates containing 25 - 250 colonies were counted and recorded as colony forming units (cfu) per gram of the fermented soymilk.

9.2.6 pH measurements

Changes in pH were monitored during fermentation of soymilk at 0, 6, 12, 24, 36 and 48 h using a pH meter (HANNA Instruments, Singapore).

9.2.7 Production of organic acids in fermenting soymilk

During culture growth, the main metabolic products are organic acids, particularly lactic and acetic acids. The concentrations of these acids were measured according to the method described in Section 3.2.5 using a high performance liquid chromatrography (HPLC). Briefly, the HPLC (Varian Associates, Walnut Creek, CA, USA) comprised of a solvent delivery system (model 9100) connecting with an auto-sampler (model 9012), a UV light detector (model 9050) and an organic acid analysis column (Aminex HPX-87H, 300 x 7.8 mm, Bio-Rad Lab, Richmond, CA, USA). The mobile phase was 0.001 M H_2SO_4 with a flow rate set at 0.6 mL/min and the temperature of the column was set at 65^{o}C. Organic acids were detected at 220 nm. For determination of organic acids, 2.5 g samples were mixed with 50 µL of 15.8 M HNO_3 and 1.0 mL of 0.001 M H_2S0_4 before subjecting a 1.5 mL aliquot of the mixture to centrifugation at 14,000 x g for 30 min at room temperature (~20^{o}C). The supernatant was filtered through a 0.45 µm membrane filter (Schleicher & Schvell, Dassel, Germany) and 20 µL of the filtrate was injected into the HPLC system.

9.2.8 Isoflavone extraction

The extraction of isoflavones from fermented and non-fermented soymilk was performed in triplicate using the method described by Otieno *et al*., (2006) with some modifications. Briefly, 50 mL aliquots of methanol were added to 1.0 g freeze-dried sample in a 150 mL round bottom flask and refluxed on a heating mantle for 1 h. The mixture was poured into a 50 mL centrifuge tube and centrifuged at 4,000 x g for 30 min at 10^{o}C using a Sorvall RT7 refrigerated centrifuge (Newtown, Conn., USA). A 2 mL aliquot of the supernatant was withdrawn and mixed with 50 μL of internal standard (ISTD) flavone solution (0.2 mg/mL) and evaporated to dryness using the speed vac concentrator connected to Savant refrigerated trap and vacuum pump (model SC110, Savant Instruments Inc., Farmingdale, NY, USA). The dried sample was dissolved in mobile phase (0.05% TFA in 50% of 100 mM ammonium acetate and 50% methanol) and the resulting solution was centrifuged at 14,000 x g for 30 min using an Eppendorf centrifuge (model 5415C; Crown Scientific). The supernatant was filtered through a 0.45 μm membrane filter (Schleicher & Schuell GmbH, Dassel, Germany) into an HPLC vial.

9.2.9 Isoflavone standards

Isoflavone standard stock solutions were prepared by dissolving genistein, daidzein, genistin and flavone in HPLC grade methanol and glycitein, daidzin and glycitin were dissolved in ethanol to give a 1 mg/mL concentration. Calibration curves were prepared for each standard with six different concentrations (0, 20, 40, 60, 80 and 100 μg/mL). A mixture of the standards was also analysed and used for identification purposes.

9.2.10 HPLC with electrochemical detection of isoflavone in fermented soymilk

For electrochemical determination of isoflavones in soymilk, a highly sensitive HPLC-ECD method was used as described previously (Klejdus *et al*., 2004) with some modifications. Isoflavone extracted from unfermented (control) and fermented soymilk samples were profiled on a reversed-phase Varian HPLC (Varian Analytical Instruments, Walnut Creek, CA, USA) system. The HPLC system for electrochemical detection consisted of the following components: a Varian model 9100 auto sampler, a Varian model 9012 solvent delivery system, injector, a Metrohm model 656 amperometric and 641 VA detector with a 1-μL cell with glassy carbon working and auxilliary electrode, and a Ag/AgCl reference electrode (Metrohm, Herisau, Switzerland). Samples were applied using a 20 μL

injection loop and separation of isoflavone compounds was achieved using an Alltima HP C18 HL, 5 μm, 250 x 4.6 mm column and a guard column (Alltech Associates, Inc., Deerfield, IL, USA). The isoflavones were eluted by isocratic runs of 40 min with mobile phase (0.05% TFA in 50% of 100 mM ammonium acetate and 50% methanol) at a flow rate of 1 mL/min and room temperature (~20^{o}C), a sensitivity range of 0.1 – 50 nA, and an electrochemical detection at +800 mV. All solvents were filtered through a 0.45 μm membrane filter (Schleicher & Schuell GmbH, Dassel, Germany) and degassed by ultrasound and He sparging.

Retention times of standards of isoflavones eluted as single peaks (glycitin, daizin, genistin, daizein, glycetein, and genistein) ranged between 2.57 - 17.48 min, and the peak areas were used for quantitation. Calibration runs were prepared for each analytical series with external standards in the range of 20 to 200 ng/mL of isoflavones. The calibration curves were linear with a regression coefficient of 0.998.

9.2.11 Statistical analysis

All results obtained were analysed as a split plot in time design using the general linear model (GLM) procedure of the SAS System (SAS, 1996). The univariate ANOVA test was validated by fulfilling the Huynh-Feldt (H-F) condition (Littell *et al*., 1998). Where appropriate, one-way ANOVA and correlational analysis were employed using Microsoft® Excel StatPro™ (Albright *et al*., 1999) and the multicomparison of means was assessed by Tukey's test. The statistical level of significance was preset at 0.05. All experiments were replicated and sub-sampled at least twice (n = 6).

9.3 RESULTS

9.3.1 Cell growth during fermentation and storage

Cell populations of *L. acidophilus* L10, *B. lactis* B94 and *L. casei* L26 in soymilk during fermentation at 6, 12, 24, 36 and 48 h at 37^{o}C are shown in Figure 9.1. The highest viable counts during fermentation occurred at 12 h for *L. casei* L26, 24 h for *B. lactis* B94, and 36 h for *L. acidophilus* L10. Subsequently the populations declined ($P > 0.05$) slowly for each microorganism after growth (Figure 9.1). Soymilk fermented with *L. acidophilus* L10, *B. lactis* B94 and *L. casei* L26 and stored at 4^{o}C for 28 d showed an increased trend of bacterial growth, with *L. acidophilus* L10 and *B. lactis* B94 exhibiting increased population by 20 and 14%, respectively (Figure 9.2). Even though the different fermentation times

influenced bacterial growth during storage at 4°C, there was a general decline in cell populations from 21 to 28 d.

9.3.2 Decline in pH during fermentation and storage

There was a decline in pH during fermentation at 6, 12, 24, 36 and 48 h at 37°C is presented in (Figure 9.3). Each batch of soymilk showed consistent decrease in pH from the initial pH of 6.58 during 48 h incubation. *B. lactis* B94 and *L. casei* L26 presented a lower but similar drop in pH (0.73 pH units) compared to *L. acidophilus* L10 which showed a decline of 0.59 pH units at the end of storage. The trend was similar and was not significantly different for samples from all 5 fermentation times during the 28 d storage period (Figure 9.4).

9.3.3 Organic acids concentration during fermentation and storage

The concentration of lactic and acetic acids in fermented soymilk produced by *L. acidophilus* L10, *B. lactis* B94 and *L. casei* L26 is shown in Figure 9.5. In general, the lactic acid concentration was higher ($P < 0.05$) than acetic acid for all treatments and times. The concentration of lactic acid in soymilk increased consistently during 48 h of fermentation for all the organisms, with *L. acidophilus* L10 producing the lowest concentration of lactic acid but not significantly ($P < 0.05$) different from *B. lactis* B94. However in comparison to *L. acidophilus* L10, the production of lactic acid by *L. casei* L26 was significantly ($P < 0.05$) higher at the end of fermentation. Even though the organic acid content increased slightly during storage at 4°C for 28 d, there was no significant difference between cultures for lactic and acetic acids (Figures 9.6 and 9.7). Since the growth was slow during storage, the production of organic acids was lower ($P > 0.05$) which resulted in a minimal decline in pH. Consequently fermented soymilk did not set during 28 d storage at 4°C.

9.3.4 β-glucosidase activity

The β-glucosidase activity of *L. acidophilus* L10, *B. lactis* B94 and *L. casei* L26 in soymilk before and after fermentation at 37°C is shown in Figure 9.8. The maximum yield of β-glucosidase activity varied ($P < 0.05$) between all three organisms. *L. acidophilus* L10 showed increased enzyme activity at 36 h of fermentation whereas *B. lactis* B94 and *L. casei* L26 attained maximum enzyme activities at 24 and 12 h respectively. During storage at 4°C, all microorganisms showed maximum β-glucosidase activity ranging from 0.117 to 0.204 μmol/mL at d 21 irrespective of fermentation time. Overall, *B. lactis* B94 exhibited the

highest β-glucosidase activity followed by *L. acidophilus* L10 during storage. The correlation between β-glucosidase activity and growth of microorganisms ranged from 0.70 for *L. acidophilus* L10 to 0.79 for *B. lactis* B94, which further suggested the effects of growth of microorganisms in soymilk on β-glucosidase activity.

9.3.5 Hydrolysis of isoflavone compounds in fermented soymilk by micro-organisms

The changes in isoflavone concentration in relation to increasing bacteria population in soymilk during 48 h fermentation at 37^{o}C with *L. acidophilus* L10, *B. lactis* B94 and *L. casei* L26, respectively, is shown in Figure 9.1. The maximum concentration of aglycones produced corresponded to the maximum cell population of each microorganism. In general, the concentration of isoflavone aglycones increased ($P < 0.05$) and at the same time, the concentration of β-glucosides were reduced ($P < 0.05$) compared to that in unfermented soymilk. However, the concentrations of malonyl- and acetyl-glucosides were relatively low and there was no significant change ($P > 0.005$) during the 48 h fermentation (Table 9.1). There was an appreciable increase in the concentration of daidzein, glycitein and genistein from 6 h of incubation and they continued to increase up to 36 h for *L. acidophilus* L10, 24 h for *B. lactis* B94 and 12 h for *L. casei* L26. *L. acidophilus* L10 yielded a 63% increase in aglycone concentration at 36 h, whereas *B. lactis* B94 and *L. casei* L26 showed 77 and 71% increase in aglycones at 24 and 12 h, of incubation, respectively compared to non fermented soymilk (Figure 9.1).

9.4 DISCUSSION

All cultures showed good growth characteristics in soymilk indicating that soymilk was a good delivery medium for probiotic organisms. Previous studies have shown that these bacteria produce α-galactosidase required to metabolise raffinose and stachyose and grow well in soymilk (Scalabrini *et al*., 1998). The growth of the organisms declined after the respective peak growth periods possibly due to diminishing nutrient supply in the medium. A similar trend of cell population decline was observed during storage at 4^{o}C for 28 d.

During soymilk fermentation, the main metabolic end products include organic acids. These lead to a reduction in the pH of soymilk. The low production of organic acids

in soymilk by LAB reported in the results offered a better environment for cell growth during fermentation and storage compared to milk. As a result, all organisms grew above 8.0 $\log_{10}$ cfu/g in fermented soymilk and this level was maintained during storage at 4^{o}C. Previous studies have reported similar findings (Angeles and Marth, 1971; Kamaly, 1997; Liu, 1997). Acetic acid is an undesirable end-product in fermented soymilk due to its 'vinegary' flavour. Therefore the high production of lactic acid over acetic acid by lactobacilli and bifidobacteria in this study was desirable for the production of fermented soymilk. Similar results were reported in Chapter 4 in soy yoghurt made with commercial soymilk and fermented by *L. acidophilus*, *Bifidobacterium* and *L. casei* and during storage at 4^{o}C.

The respective maximum β-glucosidase activities observed for each culture showed that the period of fermentation at which the enzyme activity was highest corresponded to their cell populations. The increased β-glucosidase activity between 24 and 36 h was significant ($P < 0.05$) for *L. acidophilus* L10 which had the highest β-glucosidase activity compared to *B. lactis* B94 and *L. casei* L26. Otieno *et al*. (2006) also reported that *L. acidophilus* showed the highest β-glucosidase activity at 24 h of fermentation in soymilk compared to *Bifidobacterium* spp. and *L. casei*. Generally, β-glucosidase activity was found to be strain ($P < 0.0436$) and time ($P < 0.0001$) dependent and therefore, the enzyme activity varied among the organisms used during the 48 h fermentation at 37^{o}C and this pattern continued throughout storage at 4^{o}C. *L. acidophilus*, *Bifidobacterium* and *L. casei* were reported to show strain- dependent β-glucosidase activity in soymilk (Tochikura *et al*., 1986; Otieno *et al*., 2005). The β-glucosidase enzymes produced by these microoganisms are responsible for the breakdown of β-1-6 glucosidic bond, which conjugates the pran ring of isoflavone and the sugar moieties. These enzyme activities significantly increased the content of isoflavone aglycones in fermented soymilk after incubation with *L. acidophilus*, *Bifidobacterium* and *L. casei* as compared to that in unfermented soymilk. The breakdown of isoflavone glucosides into sugar moieties and bioactive isoflavone aglycones during fermentation could improve the biological activity of soymilk.

9.5 CONCLUSIONS

L. acidophilus L10, *B. lactis* B94 and *L. casei* L26 showed varying levels of β-glucosidase activity, which depended on the growth of each organism. In addition, β-

glucosidase activity was strain dependent. *L. acidophilus* L10, *B. lactis* B94 and *L. casei* L26 showed 20, 15 and 11% greater cell counts compared to the initial numbers, respectively. Increased cell growth resulted in higher enzyme activity, which subsequently produced increased concentration of isoflavone aglycones in fermented soymilk, compared to unfermented soymilk.

Table 9.1 Changes in the concentrations (μmol per mL) of malonyl- and acetyl-glucosides undergoing fermentation in soymilk by *L. acidophilus* L10, *B. lactis* B94 and *L. casei* L26 for 6, 12, 24, 36 and 48 h at 37°C.

Probiotic bacteria	Time, h	Malonylglucosides	Acetylglucosides
L. acidophilus L10	0	0.054 ± 0.007 [a]	0.062 ± 0.004[a]
	6	0.033 ± 0.011 [bc]	0.043 ± 0.011[b]
	12	0.028 ± 0.008 [b]	0.048 ± 0.009 [b]
	24	0.016 ± 0.007 [b]	0.055 ± 0.006[ab]
	36	0.026 ± 0.005 [b]	0.055 ± 0.005[ab]
	48	0.031 ± 0.012 [b]	0.059 ± 0.005[ab]
B. lactis B94	0	0.054 ± 0.005 [a]	0.062 ± 0.003 [a]
	6	0.044 ± 0.003 [a]	0.048 ± 0.006 [b]
	12	0.030 ± 0.006 [b]	0.039 ± 0.008 [b]
	24	0.018 ± 0.006 [b]	0.038 ± 0.008 [b]
	36	0.040 ± 0.013 [a]	0.058 ± 0.011 [a]
	48	0.021 ± 0.004 [b]	0.059 ± 0.008 [a]
L. casei L26	0	0.054 ± 0.005 [a]	0.062 ± 0.003 [a]
	6	0.035 ± 0.003 [b]	0.039 ± 0.006 [b]
	12	0.018 ± 0.005 [b]	0.034 ± 0.005 [b]
	24	0.032 ± 0.011 [b]	0.050 ± 0.012 [a]
	36	0.028 ± 0.001 [b]	0.049 ± 0.003 [b]
	48	0.021 ± 0.005 [b]	0.054 ± 0.005 [a]

[abc] Means in the same column for a particular glucosides (glycosides) and strain with different small letter superscripts are significantly different; Results presented as a mean of three observations ± standard error.

Figure 9.1 a

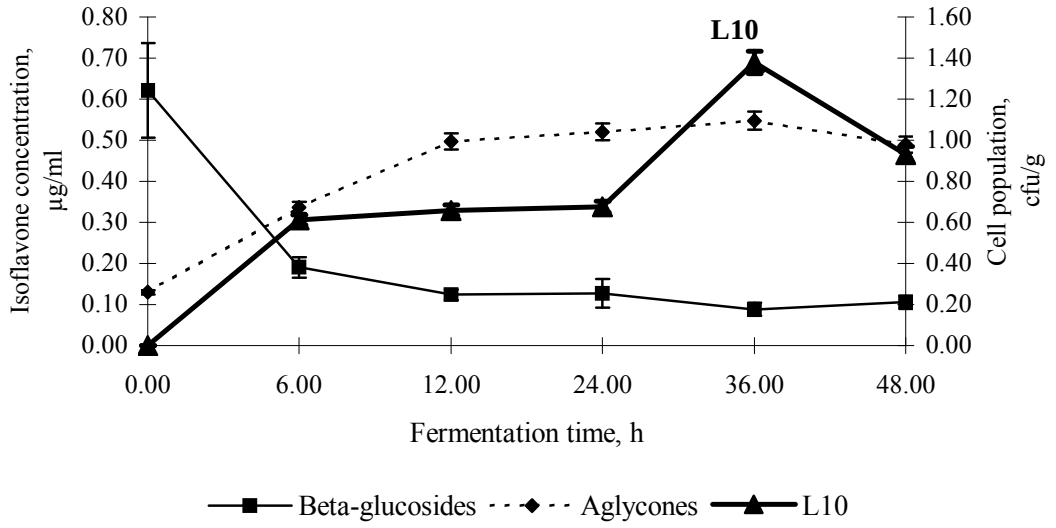

Figure 9.1 b

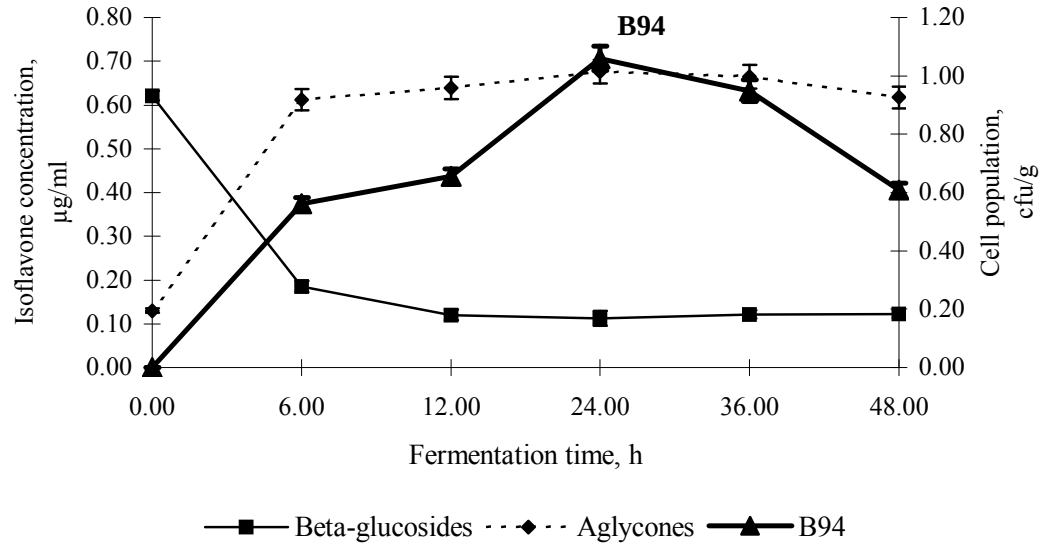

Figure 9.1 c

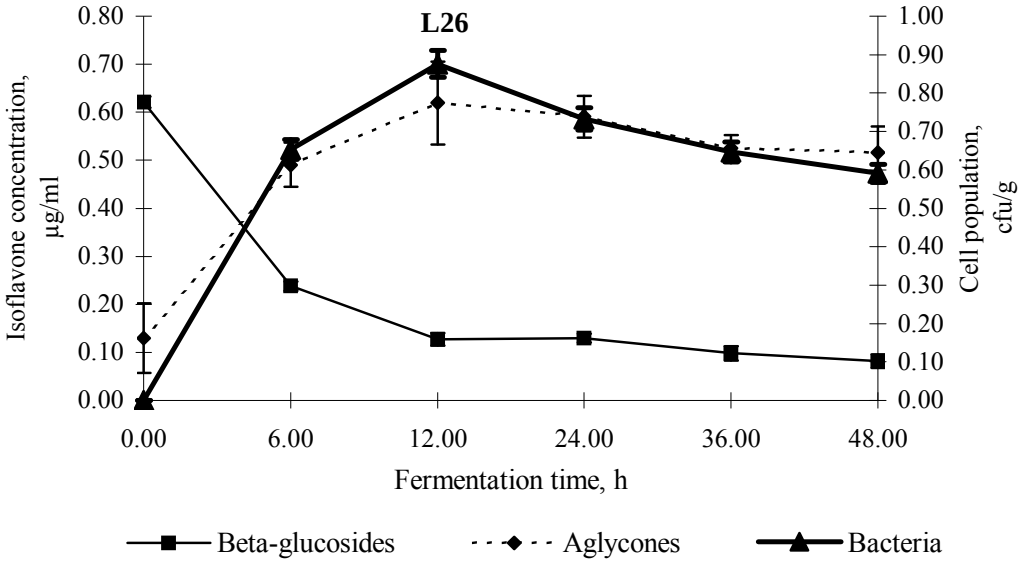

Figure 9.1 a, b & c Changes in bacteria population (Δ cfu/g) and concentrations of β-glucosides and aglycones (µg per mL) in soymilk undergoing fermentation by *L. acidophilus* L10, *B. lactis* B94 and *L. casei* L26 for 6, 12, 24, 36 and 48 h at 37°C. L10 = *L. acidophilus*, B94 = *B. lactis*, L26 = *L. casei* (n = 6, Error bars represent means ± standard error).

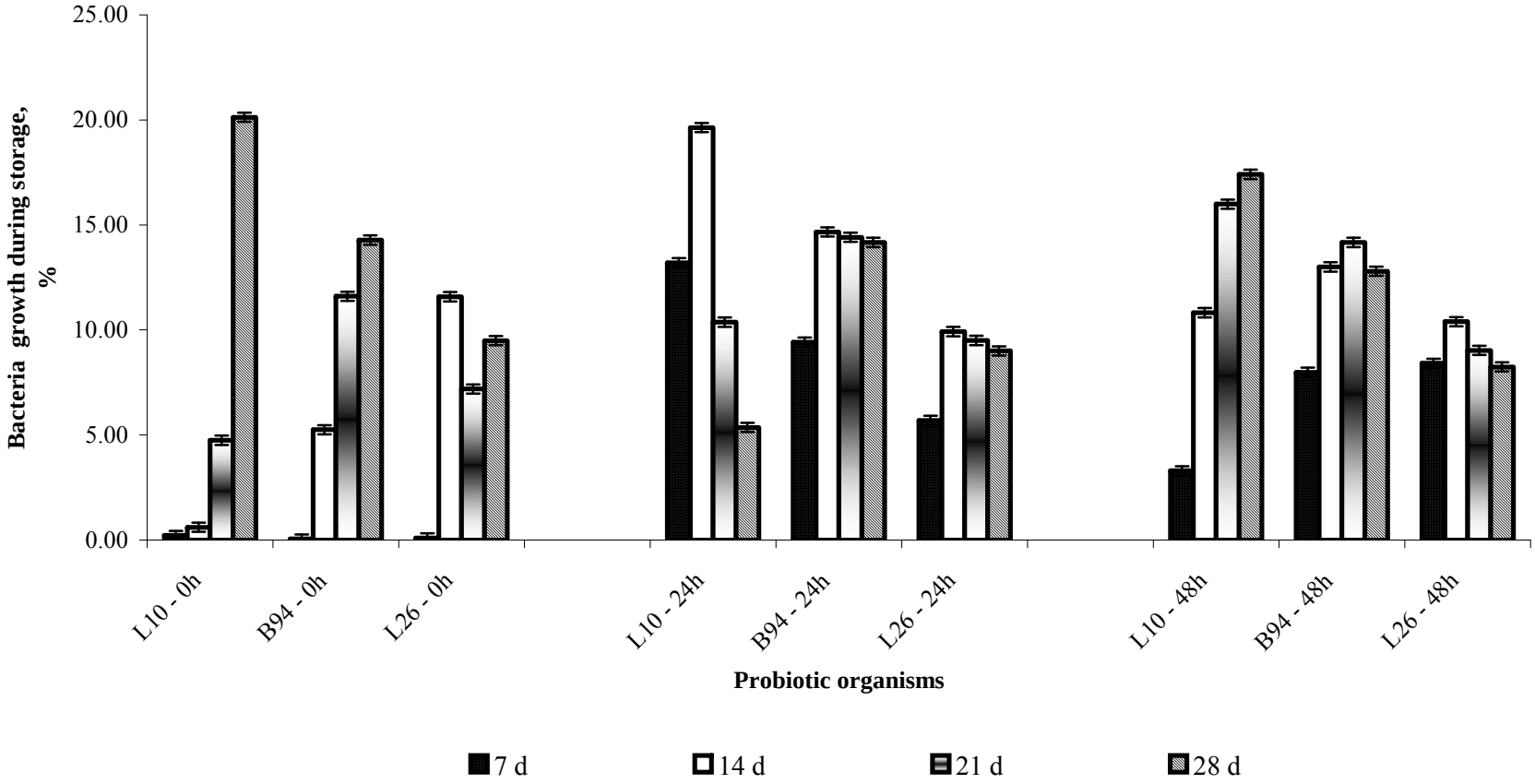

Figure 9.2 Percentage cell counts (% CFU per g) in soymilk which had undergone fermentation by *L. acidophilus* L10, *B. lactis* B94 and *L. casei* L26 for 24 and 48 h at 37°C and stored at 4°C for 28 d. L10 = *L. acidophilus*, B94 = *B. lactis*, L26 = *L. casei* (Error bars represent a pooled standard error of the mean; n = 6)

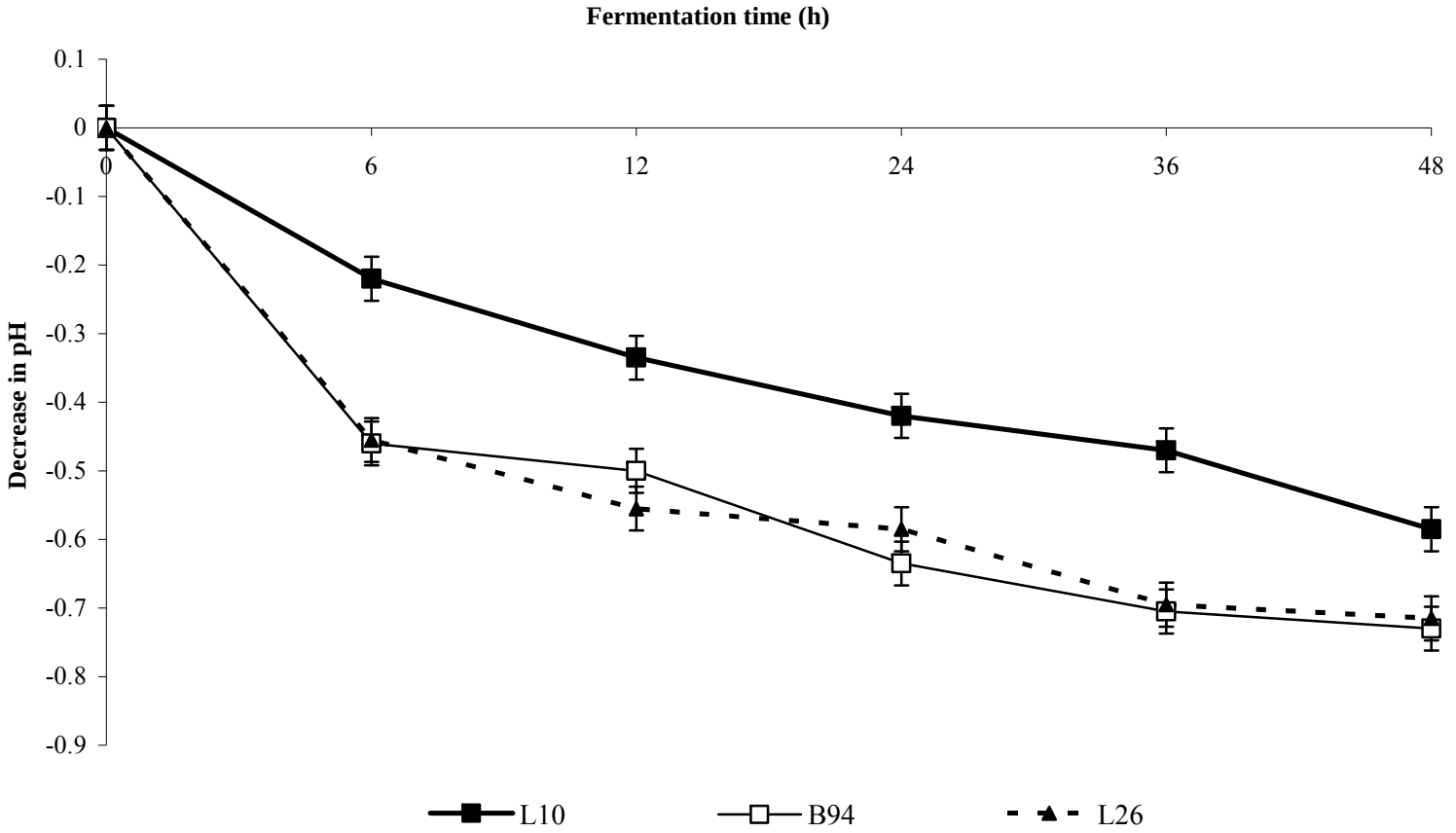

Figure 9.3 pH decline in soymilk during fermentation with *L. acidophilus* L10, *B. lactis* B94 and *L. casei* L26 for 6, 12, 24, 36 and 48 h at 37^{o}C. The negative sign indicate a reduction in pH. L10 = *L. acidophilus* L10, B94 = *B. lactis* B94, L26 = *L. casei* L26 (Error bars represent a pooled standard error of the mean; n = 6; SEM = 0.03 pH units).

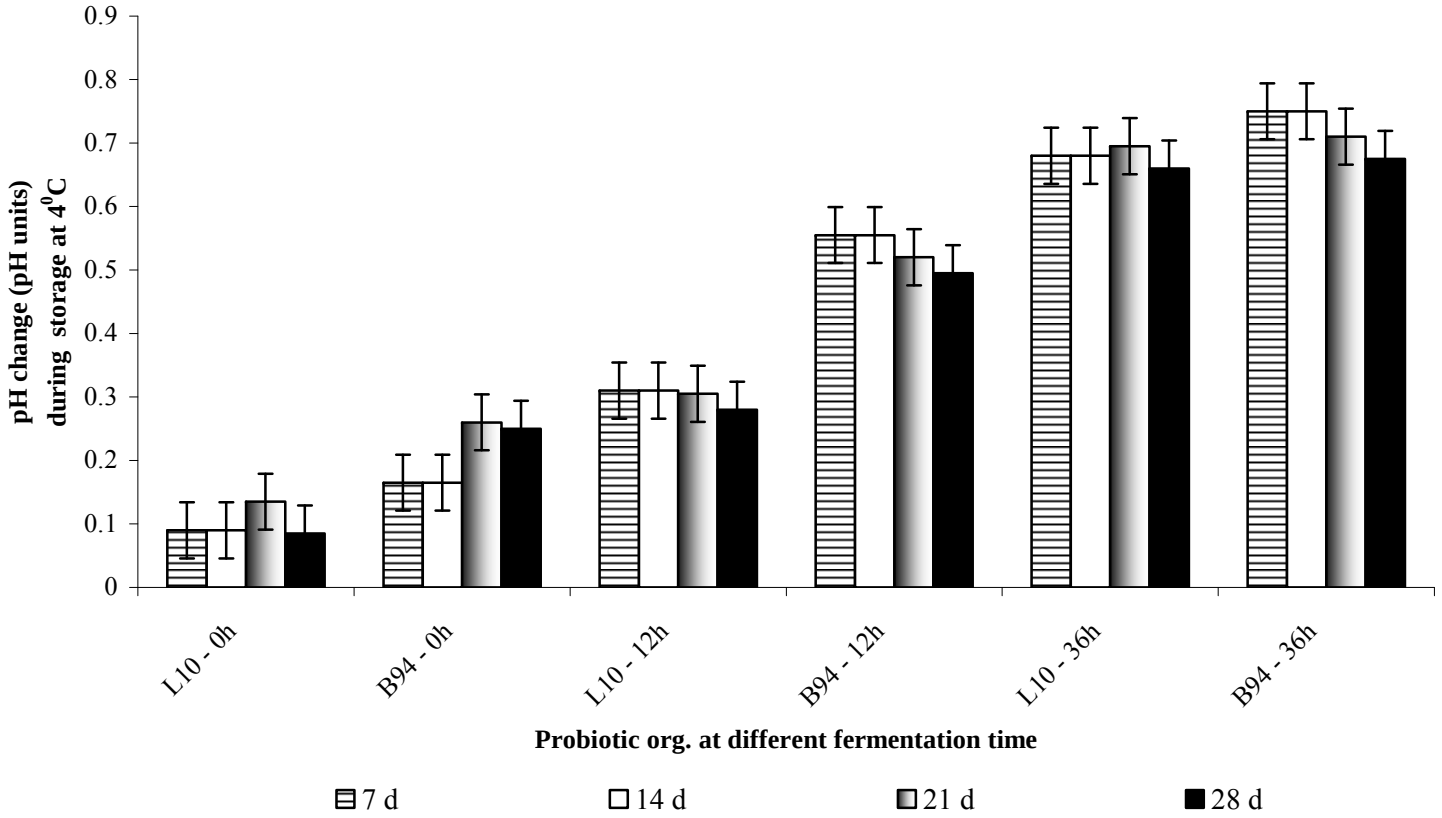

Figure 9.4. pH decline in fermented soymilk with *L. acidophilus* L10, and *B. lactis* B94 during fermentation for 36 h and storage for 28 d at 4°C. L10 = *L. acidophilus* L10, B94 = *B. lactis* B94. (Error bars represent a pooled standard error of the mean; n= 6; SEM = 0.04 pH units).

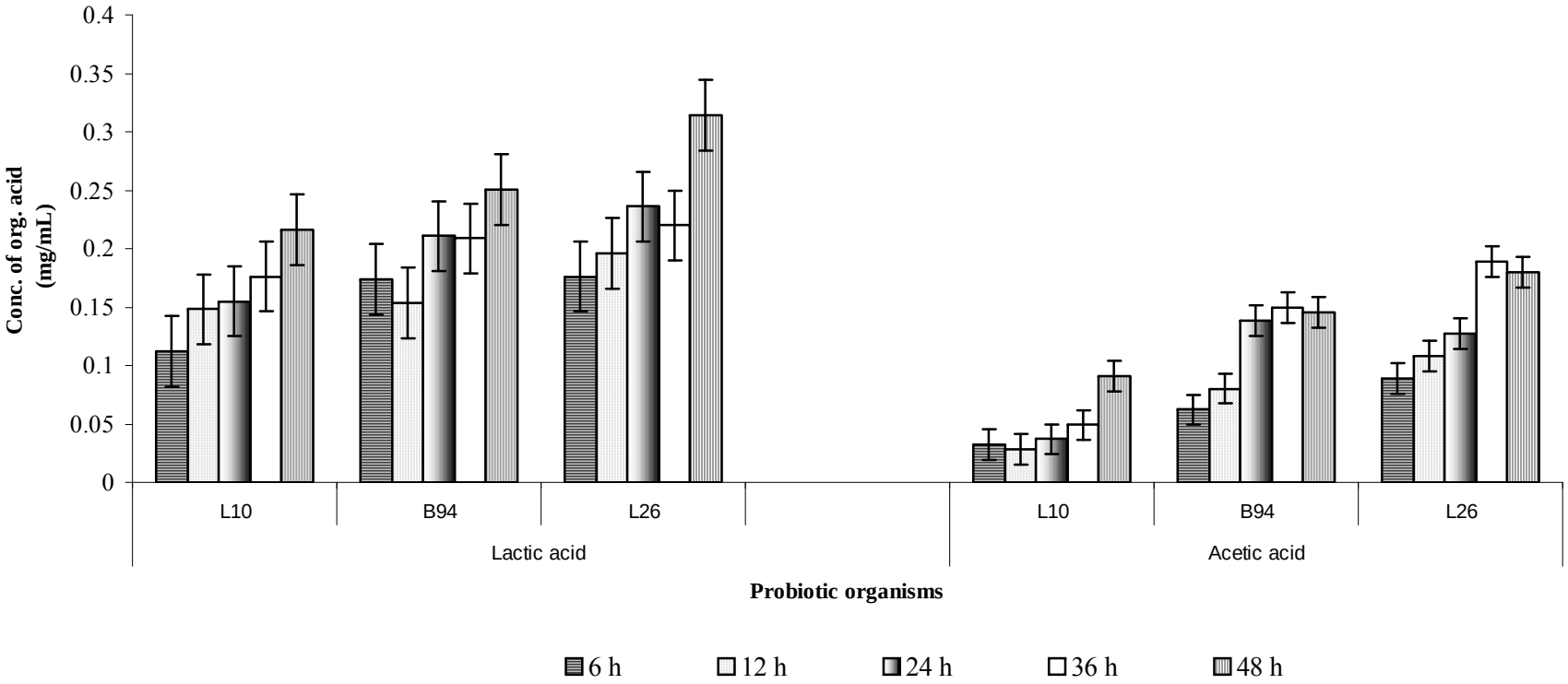

Figure 9.5. Lactic and acetic acid concentrations produced in soymilk undergoing fermentation by *L. acidophilus* L10, *B. lactis* B94 and *L. casei* L26 for 6, 12, 24, 36 and 48 h at 37°C as single cultures. L10 = *L. acidophilus*, B94 = *B. lactis*, L26 = *L. casei* (Error bars represent a pooled standard error of the mean; n = 6; SEM for lactic and acetic acid = 0.03 and 0.01 mg/ml respectively).

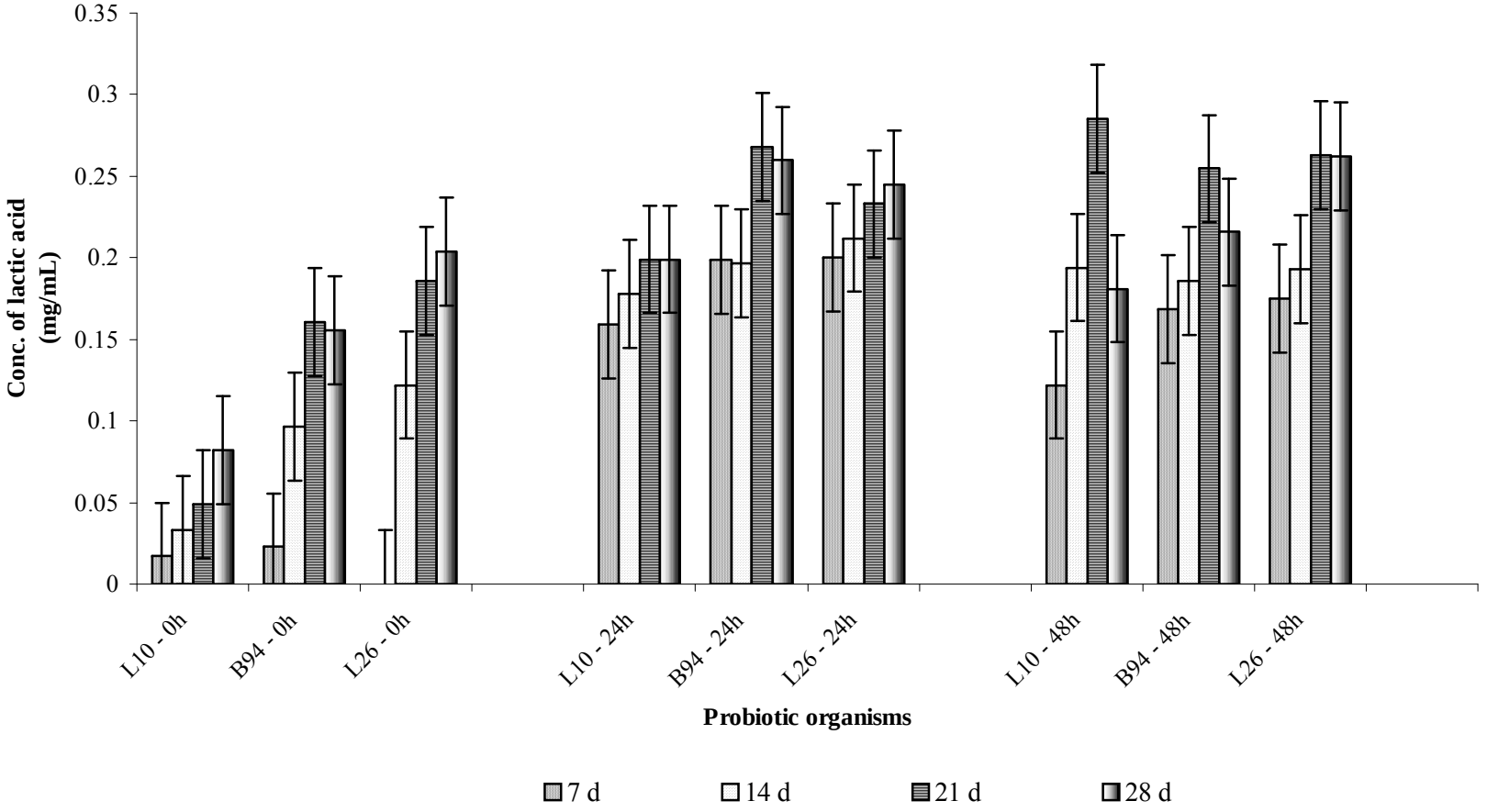

Figure 9.6 Lactic acid concentrations produced in fermented soymilk with *L. acidophilus* L10, *B. lactis* B94 and *L. casei* L26 which had undergone fermentation for 48 h at 37°C and stored for 28 d at 4°C. L10 = *L. acidophilus* L10, B94 = *B. lactis* B94, L26 = *L. casei* L26 (Error bars represent a pooled standard error of the mean; n = 6; SEM = 0.03 mg/mL).

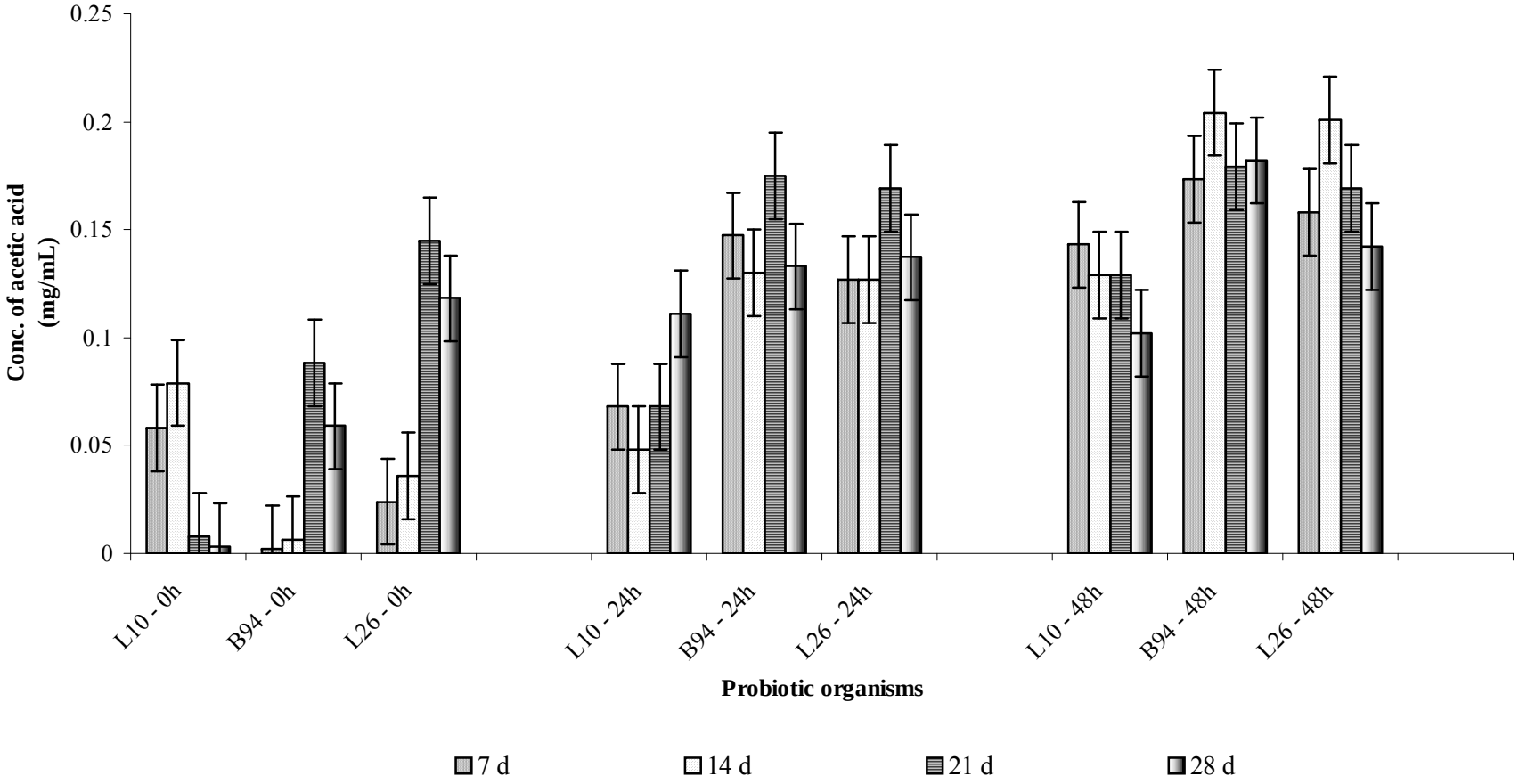

Figure 9.7 Acetic acid concentrations produced in fermented soymilk with *L. acidophilus* L10, *B. lactis* B94 and *L. casei* L26 which had undergone fermentation for 48 h at 37°C and stored for 28 d at 4°C. L10 = *L. acidophilus* L10, B94 = *B. lactis* B94, L26 = *L. casei* L26 (Error bars represent a pooled standard error of the mean; n = 6; SEM = 0.02 mg/mL).

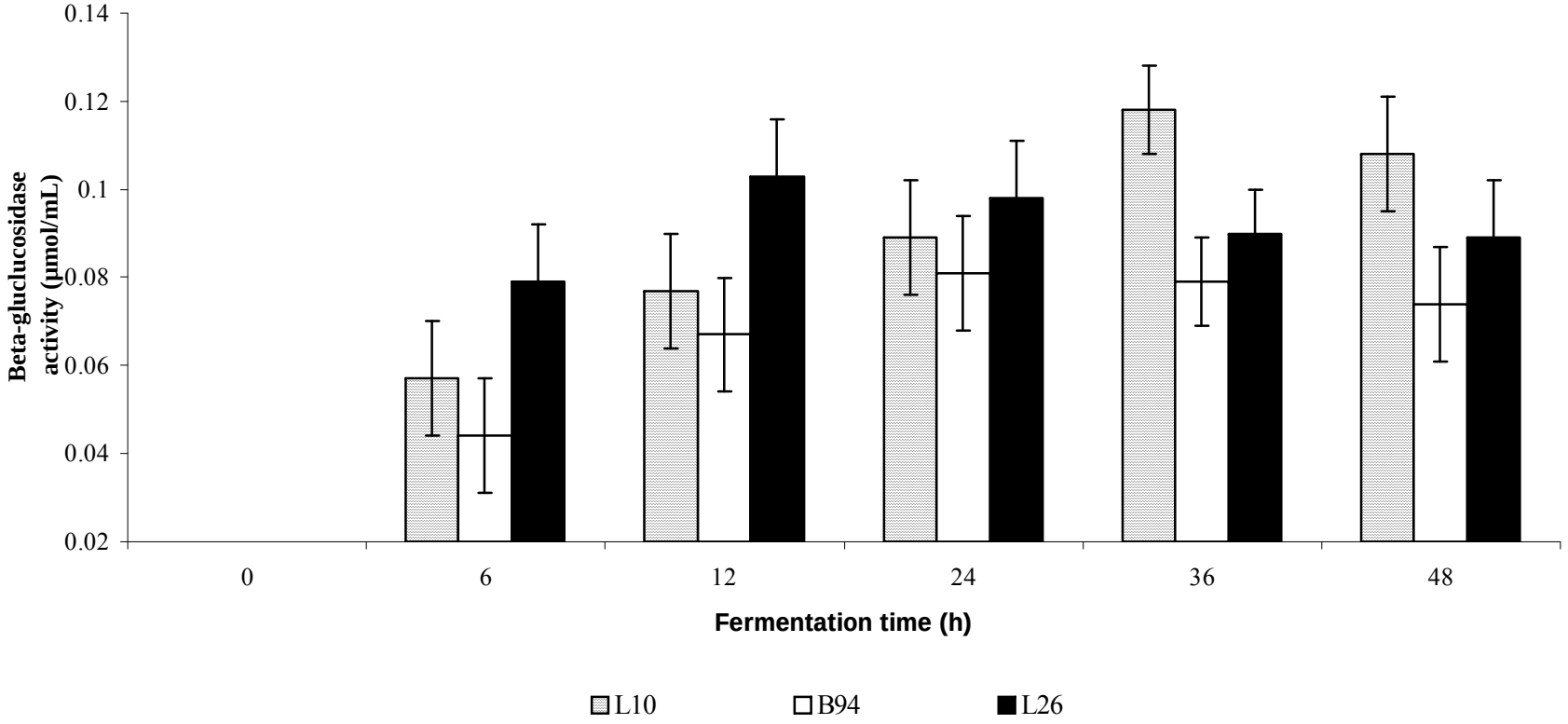

Figure 9.8. β-Glucosidase activity (µmol per mL) in soymilk undergoing fermentation by *L. acidophilus* L10, *B. lactis* B94 and *L. casei* L26 for 6, 12, 24, 36 and 48 h at 37°C as single cultures. L10 = *L. acidophilus*, B94 = *B. lactis*, L26 = *L. casei*; (Error bars represent a pooled standard error of the mean; n = 6; SEM = 0.01µmol/mL).

10.0 Rheological properties and sensory characteristics of set-type soy yoghurt

10.1 INTRODUCTION

Fermented soy products are perceived as healthy food and considered an important part of the diet. Furthermore, the incorporation of probiotic bacteria as dietary adjuncts has given rise to increased consumption of probiotic products in Europe, USA and Asia (Liu, 1997; Nagata *et al*., 1998; Kristo *et al*., 2003). Soy-based foods may provide additional benefits for the consumer due to their hypolipidemic, anticholesterolemic and anti-atherogenic properties and reduced allergenicity (Messina *et al*., 1994; Lopez-Lazaro and Akiyama, 2002). Consequently, soymilk based yoghurt offers a considerable appeal to a growing segment of consumers with certain dietary and health concerns. Probiotic bacteria are defined as "live microorganisms which when administered in adequate amounts confer health benefits on the host" (FAO/WHO, 2002). They are normally marketed as nutraceuticals in forms of capsules and powders or added to yoghurt which is a popular vehicle for incorporation of probiotic microorganisms (Lourens-Hattingh and Viljeon, 2001). The diverse range of "functional" food products currently available on the market reflects the convenience of using food as a delivery system for probiotic microorganisms (Driessen and de Boer, 1998). Much of the research concerning the health-promoting effect of soy products has focused on soy isoflavones. In addition to isoflavones, soy is an important source of many other nutrients including dietary fiber, oligosaccharides, proteins, trace minerals and vitamins, which could influence the host's well-being (Slavin *et al*., 1999). An important physiological role can be attributed to soy oligosaccharides, which could meet the standards of a prebiotic. Prebiotics have been used to promote the growth and activity of beneficial microorganisms in the large intestine (Fuller and Gibson, 1997; Gibson and Roberfroid, 1995). Some lactic acid bacteria (LAB) have been reported to grow slowly or poorly in soymilk (Mital *et al*., 1974) and produce low levels of organic acids (Liu, 1997). Therefore, to improve the growth of probiotic bacteria and production of organic acid, soymilk needs to be supplemented with various prebiotics such as raffinose or inulin or a combination of glucose and raffinose (Roberfroid *et al*., 1998; Chou and Hou, 2000; Tsangalis and Shah, 2004).

Donkor, O. N., Henriksson, A., Vasiljevic, T., & Shah, N. P. (2007). Rheological properties and sensory characteristics of set-type soy yoghurt. *Journal of Agricultural and Food Chemistry*, *55*, 9868-9876.

Soymilk, the aqueous extract of soybean, originates from Asia and presents a nutritious beverage. Western populations generally dislike the flavour profile of traditional soymilk because of its aftertaste, often described as 'beany' due to the presence of hexanal and pentanal (Pinthong *et al*., 1980). The formation of these aldehydes results mainly from the hydroperoxidation of polyunsaturated fatty acids catalyzed by lipoxygenases (Wilkens and Lin, 1970). Oriental methods of soymilk manufacture establish conditions for the oxidation to occur during the initial soaking and grinding of soybeans. Commercial methods implement steps that either prevent the formation of undesirable volatile compounds (inactivation of lipoxygenases by heating) (Liu, 1997) or remove the residual off-flavours using deodorizing techniques (Liu, 1997). Lactic acid fermentation may be used as a means to reduce beany flavours and anti-nutritional factors such as phytic acid in soybean products. Furthermore, in an attempt to reduce the aftertaste in soymilk and improve sensory attributes, sweeteners such as sucrose is added (Buono *et al*., 1990; Favaro Trindade *et al*., 2001).

The preparation of a soy yoghurt-like product with *Lactobacillus* and *Bifidobacterium* appeares to be an interesting approach to the development of new fermented soy products containing probiotic cultures reported in Chapter 4. In addition, the texture and taste of soy yoghurt are essential attributes for product acceptability by the consumers. Consequently, it is important that the probiotic culture contributes to good sensory properties of the final product (Saarela *et al*., 2000). There are very few studies on the texture of soy yoghurt containing probiotics. Supplementation with either hi-maize or inulin has been shown to affect firmness and viscosity of yoghurt as well as the growth of starter culture (Radke-Mitchell and Sandine, 1986). However, not much information is available in the literature about the effect of supplementation of inulin, raffinose or glucose on growth of microorganisms in soymilk, and rheology and sensory attributes of soy yoghurt. Rheological measurements have been widely used to characterize the structure of fermented milk/soymilk gels. The most frequently determined parameters of such testing are the elastic or storage modulus (G'), which is a measure of energy stored per oscillation cycle, the viscous or loss modulus (G"), which is a measure of the energy dissipated as heat per cycle, and the loss tangent, tan δ = G"/ G' (Kristo *et al*., 2003).

Although some published studies have dealt with yoghurt production from soymilk, not much data are available providing a complete assessment of the product including

supplementation with carbohydrates and their effects on viability of probiotic organisms, removal of aldehydes and sensory attributes and rheological properties of the product. Therefore, the aims of the present study were to examine organic acids production and aldehyde metabolism in soymilk by probiotic organisms, and to assess rheological and sensory properties of soy yoghurt during cold storage for 21 d.

10.2 MATERIALS AND METHODS

10.2.1 Substrates and chemicals

Sanitarium Organics soymilk made with 8% organic whole soybeans, raw cane sugar, food acid and mineral calcium phosphate. (Simply soy, Sanitarium, NSW, Australia), Fresh Soya Bean Drink (Fortune Beancurd Manufacturer P/L, Springvale, Vic., Australia) and inulin (Raftiline® ST, ORAFTI, Oreye, SA, Australia) were provided by the respective companies. L-cysteine.HCl, sorbitol, bacteriological agar, nalidixic acid, neomycin sulphate, lithium chloride and paromomycin sulphate (NNLP), L(+)-lactic acid, glacial acetic acid, propionic acid, butyric acid, hexanal, pentanal and D(+)-raffinose were purchased from Sigma Chemical Company (St. Louis, MO, USA). Reinforced clostridia agar (RCA), glucose, yeast extract and bacteriological peptone were purchased from Oxoid (West Heidleberg, Vic, Australia), H_2SO_4 and ethanol from Merck (Merck KGaA, Darmstadt, Germany), while de Man, Rogosa and Sharpe (MRS) agar was purchased from Amyl Media (Dandenong, Vic, Australia).

10.2.2 Bacterial strains and culture conditions

Lactobacillus acidophilus LAFTI® L10 (L10), *L. casei* LAFTI® L26 (L26) and *Bifidobacterium animalis* ssp. *lactis* LAFTI® B94 (B94) were provided by DSM Food Specialties (Moorebank, NSW, Australia). The selected probiotic microorganisms used in this study have been investigated extensively and are shown to possess several important probiotic properties (Crittenden *et al*., 2005). *Streptococcus thermophilus* St1342 and *Lactobacillus delbrueckii* ssp. *bulgaricus* Lb1466 were obtained from the Victoria University Culture Collection (Werribee, Australia). Each organism was stored at -80^{o}C prior to use. Sterile 10 mL portions of de Man, Rogosa and Sharpe (MRS) broth (Sigma) were inoculated with 1% of each organism and incubated at 42^{o}C for *L. delbrueckii* ssp. *bulgaricus* Lb1466 and 37^{o}C for *S. thermophilus* St1342, *L. acidophilus* LAFTI® L10, *B. animalis* ssp. *lactis* LAFTI® B94 and *L.*

casei LAFTI® L26. For the propagation of *Bifidobacterium*, sterile MRS broth was supplemented with 0.05% L-cysteine.hydrochloride to provide anaerobic condition and stimulate their growth (Shah and Ravula, 2000). The activated organisms after three successive transfers were used for the production of soy yoghurt. The cultures were prepared by inoculating each organism in 10 mL aliquots of sterilized Sanitarium organics soymilk supplemented with 2% (w/v) glucose and 1% (w/v) yeast extract for the manufacturing of the fermented soymilk.

10.2.3 Preparation of soy yoghurt batches

Six batches of soy yoghurt were prepared using the method described Section 4.2.2. Each batch was made with 2 L of commercial soymilk with 2% (w/v) inulin or 1% (w/v) each of raffinose and glucose or without any supplementation according to the experimental plan shown in Table 10.1. Three batches of soymilk were made with yoghurt culture only whereas the other three batches were made with yoghurt and probiotic cultures. All experiments were conducted under aseptic conditions in a laminar flow. Inoculated soymilk batches were then poured into 70 mL sterile clear/transparent plastic cups with lids (30 mL per cup) and incubated at 42^{o}C until the pH of 4.50 was reached. The products were removed from the incubator and stored at 4^{o}C for 28 d. Viability of bacteria, expressed as log cfu/mL, and metabolic activity measured as production of organic acids, aldehydes content in product and rheological properties were determined 12 h post-fermentation and subsequently at weekly intervals. Sensory evaluation of the products was assessed at day 1 and day 21 of storage at 4^{o}C.

10.2.4 Determination of viability of probiotics during cold storage

The colony counts of *S. thermophilus* St1342, *L. delbrueckii* ssp. *bulgaricus* Lb1466., *L. acidophilus* L10, *L. casei* L26 and *B. animalis* ssp. *lactis* B94 were determined just after inoculation, at the end of fermentation (final pH 4.5) and during storage at 4^{o}C according to the procedure described in Chapter 3, Section 3.2.4.

10.2.5 Measurement of pH

The pH of samples was determined using a pH meter (model 8417; HANNA Instruments, Singapore). The pH of all batches of soy yoghurt was recorded at the end of

fermentation, 12 h post-fermentation, and subsequently at weekly intervals. The measurements were performed in triplicate for each soy yoghurt sample.

10.2.6 Quantification of organic acids

The determination of lactic acid, acetic acid, propionic acid and butyric acid was performed according to procedure described in Section 3.2.5.

10.2.7 Headspace (HS) analysis of aldehydes

The levels of n-hexanal and pentanal were determined in soy yoghurt and in two types of commercial soymilk using the method of Tsangalis and Shah (2004) and Scalabrini *et al*. (1998) with some modifications. Frozen (-20^{o}C) soy yoghurt and soymilk samples were thawed and 2 g of sodium chloride was added to 5 mL of each sample in an air-tight HS bottle. The samples were heated at 40^{o}C for 45 min. Analysis was performed using a gas chromatograph equipped with a flame ionization detector (FID) (Varian) and a high resolution GC column DB-1 (29 m x 0.32 mm fused-silica column coated with a 0.25 μm film) (J & W Scientific, Folsom, CA, USA). The carrier gas was hydrogen (H_2) at 0.36 bar and a flow rate of (2.5 mL/min) was used. For the FID detector, H_2 and instrument air were maintained at the flow rates of 40 and 450 mL/min, respectively. The column was maintained at 40^{o}C during transfer of the HS gases. After 2 min, the column was programmed to heat at the rate of 7^{o}C/min to 140^{o}C, held for 1 min, then heated again at the rate of 10^{o}C/min to 230^{o}C. The HS gas injection volume (2 mL) was used for both samples and standards with retention times of 2.36 and 3.79 min for pentanal and hexanal, respectively.

10.2.8 Rheological measurements

The rheological properties of soy yoghurts were measured using a controlled-stress rheometer (Physica MCR 301, Anton Paar, GmbH, Germany), equipped with a temperature and moisture regulating hood and a cone and plate geometry (CP50-1, 50 mm diameter, 1^{o} angle and 0.02 mm gap). The temperature was regulated by a viscotherm VT 2 circulating bath and controlled with a Peltier system (Anton Paar) and kept constant at 5^{o}C with an accuracy of ± 0.1^{o}C. The data of all rheological measurements were analyzed with the supporting software Rheoplus/32 v 2.81 (Anton Paar).

10.2.9 Viscoelastic properties and flow behaviour

After gentle stirring for 10 sec with a plastic spoon, a small sample of soy yoghurt was deposited on the inset plate. After setting the module into a measuring position, the sample was pre-sheared at a preselected shear rate of 500/s to avoid any bias due to structural memory and subsequently rested for 5 min to allow for a structural rebuilding. The newly formed structure would be governed by the system composition. The sample was then subjected to a small amplitude oscillatory measurement (SAOM). The magnitude of strain was verified prior to experiments by conducting an amplitude test to ensure that the test was performed in the linear viscoelastic domain. First, a frequency sweep test was performed using a frequency ramp from 0.1 to 100 Hz at a constant strain of 0.1% to ascertain viscoelastic properties. This test was followed by a shear rate sweep by increasing the shear rate from 0 to 100/s (upward curve), followed by decreasing the shear rate from 100 to 0/s (downward curve) to assess the flow behaviour of the soy yoghurt. The time required for the upward or downward shear rate ramp was set at 3 min (for a total time of 6 min).

10.2.10 Flow behaviour model

The model used to fit the flow behaviour data was that of Ostwald de-Waele, also known as the Power law, and is represented by the equation:

$$\eta a = \frac{\tau}{\gamma} = k\gamma n^{-1}$$

where ηa is the apparent viscosity (Pa s), τ is the shear stress (Pa), γ is the shear rate (s^{-1}), k is the consistency index (Pa s*n*), and *n* is the flow behaviour index. The larger the value of k the thicker the product and therefore more viscous is the fluid. In this model the parameter *n* constitutes a physical property that characterizes a non-Newtonian behaviour and when $n < 1$, the fluid is pseudo-plastic (Bueno and Garcia-Cruz, 2001).

10.2.11 Sensory evaluation

The sensory properties of set-style probiotic and control soy yoghurt batches were evaluated by an untrained panel of 30 assessors recruited from students and staff members of

the School of Molecular Sciences, Victoria University, Werribee Campus. The ethics approval was obtained from the Human and Animal Ethics Committee of the Victoria University. The products were evaluated at day 1 and day 21 of storage at 4^{o}C. The samples were served at 7-10^{o}C in plastic cups and were coded with three-digit numbers. Water was available for panel members to rinse their pallet between samples. Crackers were also supplied to aid in removing any carry overs between tasting (Favaro Trindade *et al*., 2001). A test form comprising 5 sensory attributes namely appearance, mouthfeel, beany flavour, acid intensity and overall acceptance was given to each assessor. A structured nine point hedonic scale ranging from 'like extremely', 'extremely intense' or 'high' rated nine points, to 'dislike extremely', 'absent' or 'low' rated one point was used to numerically describe the sensory properties. Two sensory evaluation sessions were performed by the same group of panelists in order to assess the acceptability of the products compared to the controls as affected by supplementation with glucose or prebiotic and the presence of probiotic organism. The scores were analyzed statistically using two-way ANOVA test and Tukey's test for multicomparison of the means (Poste *et al*., 1991).

10.2.12 Statistical analysis

The experiments were organized as a random, full factorial design exploring the influence of probiotics cultures, supplementation with prebiotics and glucose and time as the main effects. All experiments were replicated three times and sub-sampled at least twice (n = 6). Results were analyzed as a repeated measures design using general linear model procedure of the SAS system (SAS, 1996). Where appropriate, one or two-way ANOVA and correlational analyses were employed using Microsoft® Excel StatProTM (Albright *et al*., 1999) and the multicomparison of means was assessed by Tukey's test. The level of significance was preset at $p \leq 0.05$.

10.3 RESULTS AND DISCUSSION

10.3.1 Cell growth

The viable population of each bacteria at the end of fermentation compared to their initial number is presented in Table 10.2. The increase in the number of *S. thermophilus* St1342, *L. delbrueckii* ssp. *bulgaricus* Lb1466, *L. acidophilus* L10, *L. casei* L26 and *B. animalis* B94

after fermentation and storage was defined as $\Delta N = \log_{10}N - \log_{10}N_0$, where ΔN is the increase in the number of bacteria, N_0 the number of bacteria just after inoculation of soymilk, N the number of bacteria at the end of fermentation. A high number of the probiotic culture at the end of fermentation is one of the prerequisites to maintain high levels of the probiotic organism during storage (Lourens-Hattingh and Viljeon, 2001). Hence, it was important to establish conditions that would promote growth of these microorganisms in soy yoghurt. Therefore, soymilk was supplemented with 2% inulin or 1% each of raffinose and glucose to investigate the effect of these carbohydrates on viability of probiotic organisms and results were compared to those in the soymilk without any supplementation. In an earlier study in Chapter 7, *S. thermophilus* St1342, *L. delbrueckii* ssp. *bulgaricus* Lb1466, *L. acidophilus* L10, *L. casei* L26 and *B. animalis* B94 were shown to possess α-galactosidase activity in the presence of oligosaccharides. As a result, these microorganisms showed improved growth in soymilk during fermentation and storage at 4^{o}C. Previous studies reported in Chapters 5 and 7, have also shown that the supplementation of 2% inulin or 1% each of raffinose and glucose substantially stimulated growth of probiotic bacteria in milk during fermentation at 42^{o}C and in MRS incubated at 37^{o}C for 48 h. Despite the slow growth of the bacteria in soy yoghurts, cell viability was maintained above 8 log cfu/g of product for all microorganisms throughout the storage. Similar results were reported in Chapter 4 in soy yoghurt supplemented with 1% α-lactose monohydrate.

10.3.2 pH changes

The initial pH of soymilk was 7.1 and was slightly higher than previously reported (Park *et al*., 2005) and the pH of soy yoghurts at the end of fermentation was 4.50. The changes in pH during fermentation were found to vary with the starter cultures and substrate concentration (carbon source). Fermentation was monitored up to 15 h for unsupplemented soymilk and since prolonged incubation is usually not desirable for commercial production in the yoghurt industry, supplementation to improve fermentation time may be essential.

Changes in pH of soy yoghurts during fermentation and storage is presented in Figure 10.1 The control soy yoghurt supplemented with 1% each of raffinose and glucose exhibited the highest pH decline during fermentation and storage. Similar observations reported in Chapter 7 showed MRS supplemented with a mixture of glucose and raffinose, stimulated higher ($P <$

0.05) production of lactic acid and thus lowered the pH of the medium. By the end of storage period, post acidification caused significant ($p \leq 0.05$) pH decline during storage at 4°C.

10.3.3 Organic acids

The mean concentrations (mg/mL) of organic acids in the control and probiotic soy yoghurt during storage at 4°C are shown in Table 10.3. Extent of acidification of soymilk depends on the strains of yoghurt cultures and other associated cultures employed (Sarkar, 2006). It is evident that high concentration of organic acids compared to unfermented soymilk was a result of the added starter culture. Patel *et al*. (1989) reported that better acid and flavour production was obtained in soymilk with a combination of *S. thermophilus* HST and *L. delbrueckii* ssp. *bulgaricus* LBW than that by *S. thermophilus* B 3641 and *L. delbrueckii* ssp. *bulgaricus* B 548. The concentration of lactic acid in all the products was substantially higher than the concentration of acetic, propionic or butyric acid after fermentation and during 28 d storage at 4°C. However, despite the low production of acetic acid in soy yoghurt, its concentration was considerably higher than the concentration of propionic or butyric acid in all the products (Table 10.3). The results showed that the predominant organic acids produced during fermentation of soymilk were lactic and acetic acids. Similar lactic and acetic acids production in soy yoghurt with probiotic microorganisms was reported in Chapter 4.

There were remarkable differences in lactic and acetic acids contents of the control and probiotic yoghurts (Table 10.3). Supplementation with 2% inulin and 1% each of raffinose and glucose influenced the production of lactic acid in both the control and probiotic soy yoghurts as the concentration values were substantially higher than those with no supplementation (Table 10.3). Favaro Trindade *et al*. (2001) also showed the enhancement in the acidification rate by starter cultures grown in soymilk fortified with 2% sucrose. Consequently acid-induced soymilk gel formation occurred faster in the soymilk supplemented with raffinose/glucose or inulin reaching pH of 4.5 in 5.30 and 7.30 h, respectively, compared to 10 h fermentation time for the soymilk without any supplementation. A similar pattern was observed for the control samples with fermentation time of 7.30 and 12 h, for raffinose/glucose and inulin, respectively, as compared to 15 h for control soy yoghurt without any supplementation. Favaro Trindade *et al*. (2001) suggested an incubation time of 6 h for the manufacture of soy yoghurt for the best sensory quality. The production of butyric acid or propionic acid did not depend on

supplementation and that all cultures appeared to be capable of producing butyric acid in soymilk. Similar results were reported in Chapter 5 and Fernandez-Garcia *et al*. (1998) in yoghurts supplemented with prebiotics or fiber.

10.3.4 Pentanal and *n*-hexanal content in soy yoghurt

The concentration of pentanal and *n*-hexanal before and after fermentation at d 1 and 21 of storage at 4^{o}C is shown in Figure 10.2. The results also include the analysis of the levels of pentanal and *n*-hexanal in commercial soymilk (Sanitarium organics soymilk and fresh soya bean drink). The commercial soymilk samples did not differ significantly ($P > 0.05$) in pentanal and *n*-hexanal concentrations indicating that the content of these aldehydes were not reduced substantially during manufacturing practices (Figure 10.2). Results of our study also showed that soymilk contained substantially higher levels of *n*-hexanal than pentanal. Various studies have reported similar findings (Scalabrini *et al*., 1998; Desai *et al*., 2002; Tsangalis and Shah, 2004). Pentanal and *n*-hexanal concentrations decreased significantly ($P < 0.05$) for all batches of soy yoghurt after fermentation especially those with inulin or glucose/raffinose supplementation; however, the reduction during storage was substantially higher in the batches with probiotic than in the control soy yoghurts (Figure 10.2). Probiotic organisms possess complex enzyme systems, which may be responsible for metabolizing aldehydes (Murti *et al*., 1992). Apparently *n*-hexanal was metabolized more than pentanal, likely due to its higher concentration in soymilk (Figure 10.2). Similarly, Scalabrini *et al*. (1998), Desai *et al*. (2002) and Tsangalis and Shah (2004) reported substantial metabolism of *n*-hexanal and pentanal in soymilk fermented with *Bifidobacterium* sp. for 48 h at 37^{o}C.

10.3.5 Rheological properties of soy yoghurt

Small amplitude shear stress oscillatory testing was applied to set type soy yoghurt during cold storage. The mechanical spectra of soy yoghurt with or without inulin or raffinose and glucose are represented in Figures 10.3 and 10.4. Storage (G') and loss modulus (G'') characterize the degree of solid-like (elastic) and liquid-like (viscous) character of a gel, respectively (Kristo *et al*., 2003). Products showed a clear gel-like behaviour, in which G' was higher than G'' for all samples (Figure 10.4). The storage modulus showed a weak frequency dependency. The magnitude of storage modulus of the soy yoghurt samples showed two closely

paired G' values for the six types of products (Figures 10.3 and 10.4). Control and probiotic soy yoghurts supplemented with raffinose and glucose exhibited the highest G' followed by the probiotic soy yoghurt and that supplemented with inulin and control soy yogurt and control soy yoghurt with inulin (Figure 10.3). The differences in storage modulus reflected the gelation characteristics within the different soy yoghurts. The supplementation of raffinose and glucose may have influenced the metabolic activities of the microorganisms, and may have affected the G' values. The low G' indicated by low (solid) elastic nature of control soy yoghurts with or without inulin supplementation was further confirmed by sensory evaluation (Table 10.5).

10.3.6 Flow behaviour

The values of the flow behaviour index (n) and the consistency index (k) determined graphically by plotting shear rate (γ) versus apparent viscosity (η_a) for all the soy yoghurts are presented in Table 10.4. The apparent viscosity of the soy gel produced by the bacteria was determined and the dependence of the rheological parameters was observed in the soy yoghurt in the presence or absence of supplementation. The rheological parameters n and k varied among the various treatments of soy yoghurts in the presence of the respective carbon sources at 5^oC. The experimental data fitted well to the Power law with R^2 values ranging from 0.98 to 0.99 for the ascending curves and 0.94 to 0.99 for the descending curves. High k values and low n values ($n < 1$) were recorded for all soy yoghurts which indicated that the products were more viscous and had pseudo-plastic properties (Table 10.4). Similar rheological behaviour was reported by Bueno and Garcia-Cruz (2001) in fermented broth with polysaccharide-producing microorganisms in the presence of glucose and sucrose.

In relation to the carbon sources used, it appeared the presence of supplementation did not have significant ($P > 0.05$) effect on k for both upward and downward curves compared to probiotic soy yoghurt without any supplementation. There were also no significant ($P > 0.05$) changes in the k values for the control soy yoghurts irrespective of supplementation, which was opposite from higher ($P < 0.05$) k values observed for probiotic soy yoghurt. A higher consistency index values may be attributed to the metabolic activity of probiotic organisms which contributed to greater pseudo-plastic properties of newly formed gels. Our findings contradicted those of Bueno and Garcia-Cruz (2001) and Charles (1978) who reported that the cellular contribution in fermented broths was insignificant.

10.3.7 Sensory evaluation

The sensory ratings of individual products for appearance, mouthfeel, beany flavor, acidity and overall likeness are presented in Table 10.5. The treatment effect was associated with the supplementation used in every case with the exception of the soymilk without any supplementation. The acid character described by flavour was associated with different bacterial growth as well as supplementation in some cases. For example, probiotic fermented soymilk was more acidic than the corresponding product made with yoghurt culture only. La Torre *et al*. (2003) made similar observations in yoghurts made with commercial probiotic and starter cultures. For the appearance of all the products with the exception of control soymilk without supplementation, which apparently had a weak gel after fermentation and during storage, 36.7% of panelists declared 'liked very much' (Table 10.5). In addition, panelists observed that all the soy yoghurts did not show syneresis after fermentation and during storage at 4^{o}C. Liu (1997) also reported of appearance and physical property acceptance of soy yoghurt by consumer panels. This was in contrast to Favaro Trindade *et al*. (2001) who reported excessive syneresis in soy yoghurt produced with 5^{o} Brix soymilk after 20 h and suggested the likely cause to be the use of 13 MPa of pressure for homogenization. Panelists recorded significant differences ($P < 0.05$) in mouthfeel among the products and selected control soy yoghurts supplemented with inulin, raffinose and glucose, or probiotic soy yoghurt supplemented with raffinose and glucose over probiotic soy yoghurt with no supplementation as having better mouthfeel (Table 10.5). Soy yoghurts exhibited more beaniness in the first day than that at 21 days of storage. On the other hand, plain soy yoghurts (control and probiotic) registered substantially higher aftertaste characteristics (Table 10.5). The reduction in the beaniness of the products could be the result of bacterial metabolism of alkylic aldehydes due to better growth in the presence of supplementation. However, the absence of sweetners in the products made the presence of residual beaniness detectable by the panelists. Previous studies have used sucrose as sweetener to mask the beany flavour detected in soy derived products (Buono *et al*., 1990; Favaro Trindade *et al*., 2001). GC analysis similarly showed a significant reduction in the aldehydes content at d 21 compared to d 1 (Figure 10.2). Furthermore the panelists recorded high acidity ($P < 0.05$) in the probiotic soy yoghurts especially in those supplemented with inulin or raffinose and glucose than the control soy yoghurts (Table 10.5). The possible explanation

could be that the supplementation promoted bacterial growth and increased organic acids production (Table 10.3).

The percentage of panelists response to the sensory evaluation with respect to the various attributes is presented in Table 10.6. At the first day of storage at 4^{o}C, the mean score values for the probiotic and control soy yoghurt samples were 4.55 and 5.35, which corresponded to 'dislike slightly' and 'neither like or dislike' ratings. For the control, 31.1% of panelists declared 'like slightly' and 18.9% 'neither like or dislike'. The corresponding ratings for the probiotic products being 26% 'like slightly' and 18.9% 'neither like or dislike'. Storage for 21 days did not bring any significant changes in the evaluation scores of the control soy yoghurts, but substantial changes were detected by panelists in the probiotic soy yoghurts (Table 10.6). In this case, the distribution of acceptability responses for the control products were 30.9% 'like slightly' and 21.4% 'neither like or dislike,' whereas for the probiotic soy yoghurts the distribution was 'like slightly' 21.3%, 'neither like or dislike' 19.8%, 'dislike slightly' 25.3%, and 'dislike very much' 21.3%. Overall, based on the acceptability mean scores, the control soy yoghurt appeared to be acceptable by the consumer panel as opposed to the slight dislike of the probiotic soy yoghurt. The slight dislike of the probiotic soy yoghurt could likely be due to high production of organic acids by probiotic organisms. Adoption of recommended techniques to eliminate the anti-nutritional factors of soy bean, use of sweetening agents, manipulation of starter combinations and addition of flavours are recommended to overcome the problem of objectionable beany flavour in the product (Sarkar, 2006).

10.4 CONCLUSION

The starter cultures used in the production of soy yoghurts played an important role in acid production and flavour of the product. The addition of inulin, raffinose or glucose to soy yoghurt effectively overcame the low acid production frequently faced in soy-based yoghurt and promoted bacterial cell growth. Furthermore, the probiotic bacteria metabolized alkylic aldehydes that are responsible for beany flavour and produced low concentrations of acetic acid which provides a vinegary flavour. The appropriate carbohydrate concentrations in soymilk can improve the texture quality as well as sensory characteristics without sacrificing flavour quality. Overall, based on the acceptability mean scores, the control soy yoghurt appeared to be more acceptable by the consumer panel (30.9% 'like slightly') than probiotic soy yoghurt (25.3%

'dislike slightly'). However, in terms of mouthfeel, the supplemented products either with inulin or raffinose/glucose in control and probiotic soy yoghurts were acceptable. Rheological tests have shown that all soy yoghurt samples irrespective of supplementation, exhibited solid-like gel characteristics but supplementation with raffinose/glucose produced firmer soy yoghurts. The probiotic soy yoghurts showed more viscous and pseudo-plastic properties than the control soy yoghurts. The overall visual appearance, and rheological properties are important physical attributes, which contributed to the overall sensory perception and functionality of these products.

10.1 Experimental plan

Soy yoghurt	Supplementation	Culture
Control	None	*L. delbrueckii* ssp. *bulgaricus* Lb1466 and *S. thermophilus* St1342
	2% Inulin	*L. delbrueckii* ssp. *bulgaricus* Lb1466 and *S. thermophilus* St1342
	1% Raffinose & 1% glucose	*L. delbrueckii* ssp. *bulgaricus* Lb1466 and *S. thermophilus* St1342
Probiotic	None	*L. delbrueckii* ssp. *bulgaricus* Lb1466 and *S. thermophilus* St1342, *L. acidophilus* L10, *L. casei* L26 and *B. animalis* B94
	2% Inulin	*L. delbrueckii* ssp. *bulgaricus* Lb1466 and *S. thermophilus* St1342, *L. acidophilus* L10, *L. casei* L26 and *B. animalis* B94
	1% Raffinose & 1% glucose	*L. delbrueckii* ssp. *bulgaricus* Lb1466 and *S. thermophilus* St1342, *L. acidophilus* L10, *L. casei* L26 and *B. animalis* B94.

Table 10.2 Population of bacteria in soy yoghurt batches before and after fermentation and during storage at 4°C for 28 days

Treatment	Microorganism	Storage period, d					
		0	1	7	14	21	28
		---------- cfu/g of the product ----------					
C - Soymilk	*S. thermophilus* St 1342	7.57^{Aa}	8.63^{Bb}	8.66^{Cb}	8.54^{Ab}	8.64^{Cb}	8.76^{Ab}
	L. delbrueckii ssp. *bulgaricus* Lb 1466	7.51^{Aa}	8.68^{Bb}	8.63^{Cb}	8.58^{Ab}	8.67^{Cb}	8.83^{Ab}
C - Soymilk + 2% inulin	*S. thermophilus* St 1342	7.51^{Aa}	8.90^{Ab}	8.67^{Cb}	8.64^{Ab}	8.71^{Ab}	8.91^{Ab}
	L. delbrueckii ssp. *bulgaricus* Lb 1466	7.51^{Aa}	8.76^{Ab}	8.74^{Cb}	8.60^{Ab}	8.73^{Ab}	8.82^{Ab}
C - Soymilk + 1%raffinose & 1%glucose	*S. thermophilus* St 1342	7.54^{Aa}	9.15^{Cb}	9.09^{Ab}	9.20^{Bb}	9.18^{ABb}	9.29^{Bbc}
	L. delbrueckii ssp. *bulgaricus* Lb 1466	7.52^{Aa}	9.14^{Cb}	9.06^{Ab}	9.16^{Bb}	9.15^{ABb}	9.28^{Bbc}
P - Soymilk	*S. thermophilus* St 1342	7.45^{Aa}	8.86^{Ab}	8.93^{Ab}	8.82^{Ab}	8.72^{Ab}	9.09^{Ab}
	L. delbrueckii ssp. *bulgaricus* Lb 1466	7.43^{Aa}	8.81^{Ab}	8.92^{Ab}	8.66^{Ab}	8.92^{Ab}	8.91^{Ab}
	L. acidophilus L10	7.54^{Aa}	8.93^{Ab}	8.94^{Ab}	8.82^{Ab}	9.00^{Ab}	9.04^{Ab}
	L. casei L26	7.53^{Aa}	8.89^{Ab}	8.93^{Ab}	8.83^{Ab}	8.96^{Ab}	9.06^{Ab}
	B. animalis ssp. *lactis* B94	7.18^{Aa}	8.78^{Ab}	8.98^{Ab}	8.86^{Ab}	9.09^{Abc}	8.99^{Ab}
P - Soymilk + 2% inulin	*S. thermophilus* St 1342	7.52^{Aa}	9.08^{Ab}	9.21^{Bb}	8.87^{Abd}	8.83^{Abc}	9.10^{Ab}
	L. delbrueckii ssp. *bulgaricus* Lb 1466	7.42^{Aa}	8.98^{Ab}	9.25^{Bbc}	8.76^{Abd}	9.06^{Ab}	8.92^{Abd}
	L. acidophilus L10	7.55^{Aa}	9.06^{Ab}	9.18^{Bb}	8.86^{Abc}	9.12^{ABb}	9.06^{Ab}
	L. casei L26	7.47^{Aa}	9.04^{Ab}	9.24^{Bbc}	8.90^{Ab}	9.04^{Ab}	9.04^{Ab}
	B. animalis ssp. *lactis* B94	7.25^{Aa}	8.93^{Ab}	9.24^{Bbc}	8.83^{Abd}	9.12^{ABbce}	9.03^{Abc}
P - Soymilk + 1%raffinose & 1%glucose	*S. thermophilus* St 1342	7.48^{Aa}	9.06^{Ab}	9.04^{Ab}	8.83^{Abd}	8.97^{Abcd}	9.13^{Abc}
	L. delbrueckii ssp. *bulgaricus* Lb 1466	7.39^{Aa}	9.02^{Ab}	9.07^{Ab}	8.66^{Abc}	9.14^{ABb}	8.99^{Ab}
	L. acidophilus L10	7.45^{Aa}	9.07^{Ab}	8.99^{Ab}	8.80^{Ab}	9.04^{Ab}	9.11^{Ab}
	L. casei L26	7.51^{Aa}	9.07^{Ab}	9.03^{Ab}	8.83^{Abc}	9.12^{ABb}	9.13^{Ab}
	B. animalis ssp. *lactis* B94	7.25^{Aa}	8.93^{Ab}	9.03^{Ab}	8.67^{Abc}	9.09^{Ab}	8.85^{Abc}
SEM				0.08			

^{abcde} Means in the same row with different small letter superscripts are significantly different; ^{ABC} Means in the same column with different capital letter superscripts are significantly different; C = Control; P = probiotic.

Table 10.3 Organic acid contents of soy yoghurt made with or without probiotics during storage at 4°C

Treatment	Storage time, d	Organic acids, mg/mL			
		Lactic	Acetic	Propionic	Butyric
C - Soymilk	0	0.28[a]	0.06[a]	0.02[a]	0.03[a]
	1	3.65[b]	1.19[b]	0.05[ab]	0.45[b]
	7	3.97[b]	1.37[b]	0.06[b]	0.49[b]
	14	3.94[b]	1.12[bd]	0.07[b]	0.56[b]
	21	4.02[c]	1.49[c]	0.07[b]	0.47[b]
	28	5.84[d]	1.47[c]	0.10[c]	0.82[bc]
C - Soymilk + 2% inulin	0	0.27[a]	0.06[a]	0.04[a]	0.06[a]
	1	3.95[b]	0.99[b]	0.05[a]	0.58[b]
	7	4.24[b]	1.08[b]	0.06[a]	0.66[b]
	14	3.01[b]	1.31[c]	0.06[a]	0.66[b]
	21	4.09[b]	1.30[c]	0.06[a]	0.66[b]
	28	4.19[b]	1.29[c]	0.06[a]	0.71[b]
C - Soymilk + 1%raffinose & 1%glucose	0	0.26[a]	0.10[a]	0.04[a]	0.06[a]
	1	6.20[b]	0.28[a]	0.06[ab]	0.06[a]
	7	7.49[b]	0.28[a]	0.07[ab]	0.08[a]
	14	8.27[c]	0.33[b]	0.08[ab]	0.09[a]
	21	9.39[d]	0.40[b]	0.10[b]	0.08[a]
	28	10.03[d]	0.35[b]	0.10[b]	0.08[a]
P - Soymilk	0	1.48[a]	0.11[a]	0.09[a]	0.09[a]
	1	6.60[c]	0.93[b]	0.09[a]	0.52[b]
	7	8.70[c]	0.97[b]	0.10[a]	0.58[b]
	14	9.09[c]	1.01[b]	0.11[a]	0.59[b]
	21	9.47[c]	1.11[bc]	0.11[a]	0.64[c]
	28	11.25[d]	1.23[c]	0.12[a]	0.58[b]
P - Soymilk + 2% inulin	0	1.37[a]	0.09[a]	0.09[a]	0.12[a]
	1	7.27[b]	0.58[b]	0.10[a]	0.33[b]
	7	8.70[c]	0.55[b]	0.10[a]	0.40[b]
	14	10.36[d]	0.76[c]	0.12[a]	0.48[b]
	21	10.97[d]	0.77[c]	0.13[a]	0.57[b]
	28	11.62[d]	0.69[b]	0.13[a]	0.59[bc]
P - Soymilk + 1%raffinose & 1%glucose	0	1.50[a]	0.10[a]	0.08[a]	0.12[a]
	1	7.29[b]	0.21[a]	0.08[a]	0.31[b]
	7	8.92[c]	0.23[a]	0.11[a]	0.41[b]
	14	10.20[c]	0.25[a]	0.11[a]	0.48[b]
	21	10.41[d]	0.34[b]	0.11[a]	0.56[b]
	28	11.28[d]	0.23[a]	0.12[a]	0.59[bc]
SEM		0.49	0.07	0.01	0.09

[abcd] Means in the same column per treatment with different small letter superscripts are significantly different; P = probiotic; C = Control

Table 10.4 Flow behavior of soy yoghurt at 5°C after ten days of storage at 4°C

Treatment	Up			Down		
	k (Pa s^n)	n	R^2	k (Pa s^n)	n	R^2
C - Soymilk	9.67^{b}	0.21^{a}	0.98	6.33^{a}	0.32^{a}	0.99
C - Soymilk + 2% inulin	10.05^{b}	0.19^{b}	0.99	6.54^{a}	0.32^{ab}	0.94
C - Soymilk + 1%raffinose & 1%glucose	9.98^{b}	0.20^{a}	0.98	6.84^{a}	0.31^{ab}	0.99
P - Soymilk	12.87^{a}	0.18^{b}	0.98	8.54^{a}	0.26^{b}	0.99
P - Soymilk + 2% inulin	10.51^{b}	0.20^{a}	0.98	7.14^{a}	0.31^{ab}	0.99
P - Soymilk + 1%raffinose & 1%glucose	10.24^{b}	0.20^{a}	0.98	6.99^{a}	0.32^{ab}	0.99
SEM	0.53	0.004	0.00	0.78	0.01	0.00

[ab] Means in the same column with different small letter superscripts are significantly different; C = Control; P = probiotic.

Table 10.5 Results of sensory evaluation (n = 30) of soy yoghurt with different supplementation during storage at 4°C

	Treatments					
Attributes	C - Soymilk	C - Soymilk + 2% inulin	C - Soymilk + 1%raffinose & 1%glucose	P - Soymilk	P - Soymilk + 2% inulin	P - Soymilk + 1%raffinose & 1%glucose
Day 1						
Appearance	5.62 ± 0.15^{b}	6.91 ± 0.15^{a}	6.86 ± 0.15^{a}	6.86 ± 0.15^{a}	6.06 ± 0.15^{ab}	6.59 ± 0.15^{a}
Mouthfeel	5.62 ± 0.13^{bc}	5.71 ± 0.13^{bc}	6.42 ± 0.13^{a}	4.95 ± 0.13^{bcd}	4.91 ± 0.13^{bcd}	6.19 ± 0.13^{ab}
Beany flavour	6.55 ± 0.13^{a}	6.46 ± 0.13^{a}	6.24 ± 0.13^{a}	6.28 ± 0.13^{a}	5.75 ± 0.13^{b}	5.17 ± 0.13^{b}
Acidity	3.08 ± 0.16^{bc}	3.04 ± 0.16^{bc}	6.19 ± 0.16^{a}	5.22 ± 0.16^{b}	6.15 ± 0.16^{a}	5.88 ± 0.16^{a}
Overall likeness	5.08 ± 0.13^{b}	5.62 ± 0.13^{a}	5.35 ± 0.13^{ab}	4.37 ± 0.13^{c}	4.15 ± 0.13^{c}	5.13 ± 0.13^{ab}
Day 21						
Appearance	4.25 ± 0.17^{c}	6.43 ± 0.17^{ab}	6.01 ± 0.17^{b}	6.91 ± 0.17^{a}	5.95 ± 0.17^{b}	6.11 ± 0.17^{b}
Mouthfeel	5.69 ± 0.11^{a}	5.74 ± 0.11^{a}	5.69 ± 0.11^{a}	4.30 ± 0.11^{bc}	5.15 ± 0.11^{b}	4.89 ± 0.11^{b}
Beany flavour	6.16 ± 0.10^{a}	5.85 ± 0.10^{b}	5.80 ± 0.10^{b}	5.74 ± 0.10^{b}	5.37 ± 0.10^{c}	5.11 ± 0.10^{c}
Acidity	3.18 ± 0.14^{bc}	3.72 ± 0.14^{bc}	6.54 ± 0.14^{b}	7.76 ± 0.14^{a}	7.55 ± 0.14^{a}	7.39 ± 0.14^{a}
Overall likeness	5.90 ± 0.10^{a}	5.74 ± 0.10^{a}	4.94 ± 0.10^{b}	3.56 ± 0.10^{bc}	4.30 ± 0.10^{bcd}	3.93 ± 0.10^{bc}

C = Control with *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus* only; P = probiotic with (*L. delbrueckii* ssp. *bulgaricus*, *S. thermophilus*), *L. acidophilus*, *B. animalis* ssp. *lactis*, and *L. casei*; ^{abcd} Means in the same row with different small letter superscripts are significantly different.

Table 10.6 Percentage of panelists' response to the sensory attributes in soy yoghurt during storage at 4^{o}C

Overall likeness	% of Panelist response	
	Control soy yoghurt	Probiotic soy yoghurt
Day 1		
Like extremely	3.33	0.00
Like very much	15.56	6.67
Like slightly	31.11	26.67
Neither like or dislike	18.89	22.22
Dislike slightly	18.89	20.00
Dislike very much	10.00	20.00
Dislike extremely	2.22	4.44
Total	100	
Day 21		
Like extremely	2.67	0.00
Like very much	17.33	4.00
Like slightly	30.67	16.00
Neither like or dislike	24.00	17.33
Dislike slightly	17.33	30.67
Dislike very much	8.00	22.67
Dislike extremely	0.00	9.33
Total	100	

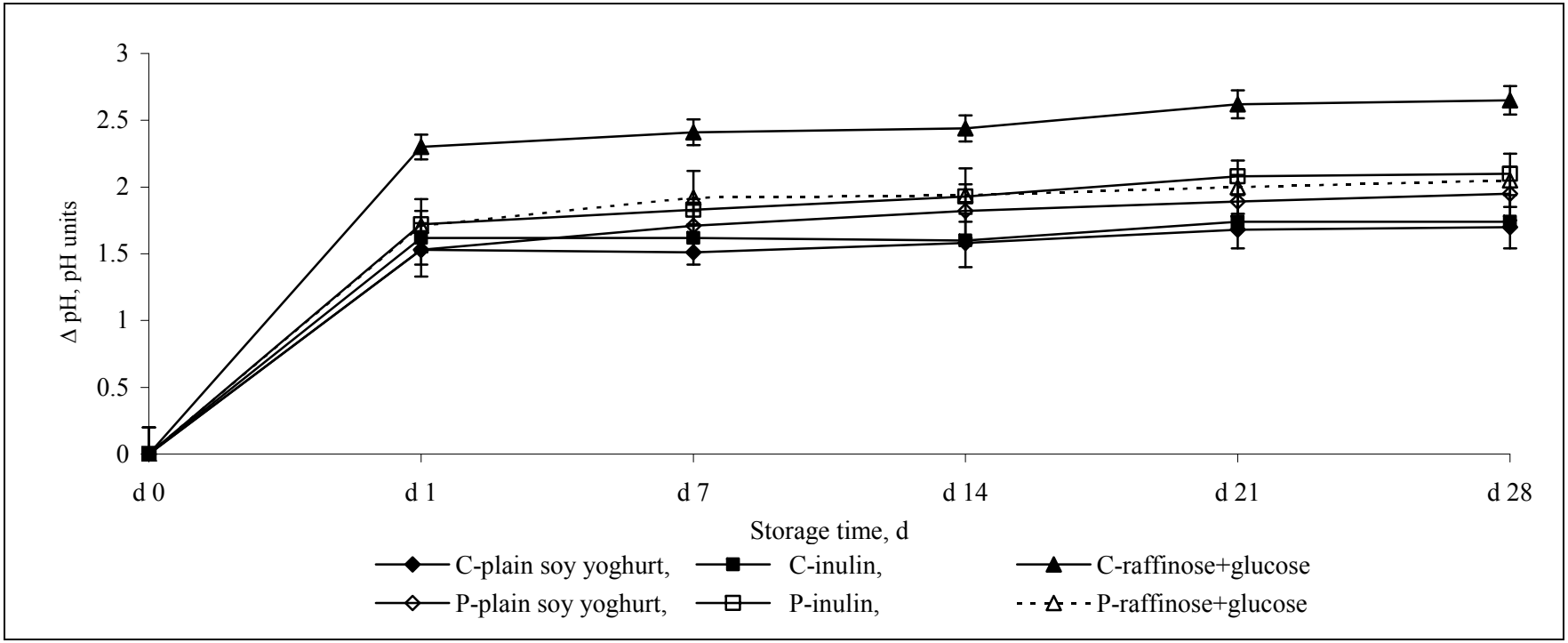

Figure 10.1 pH changes (Δ pH) during manufacture and storage of plain soy yoghurt and soy yoghurt supplemented with 2% inulin or 1% each of raffinose and glucose. C = Control soy yoghurt with only *S. thermophilus* St1342 and *L. delbrueckii* ssp. *bulgaricus* Lb 1466; P = Probiotic soy yoghurt with *S. thermophilus* St1342, *L. delbrueckii* ssp. *bulgaricus* Lb 1466, *L. acidophilus* L10, *B. animalis* ssp *lactis* B94 and *L. casei* L26 (Error bars present a pooled standard error of the mean).

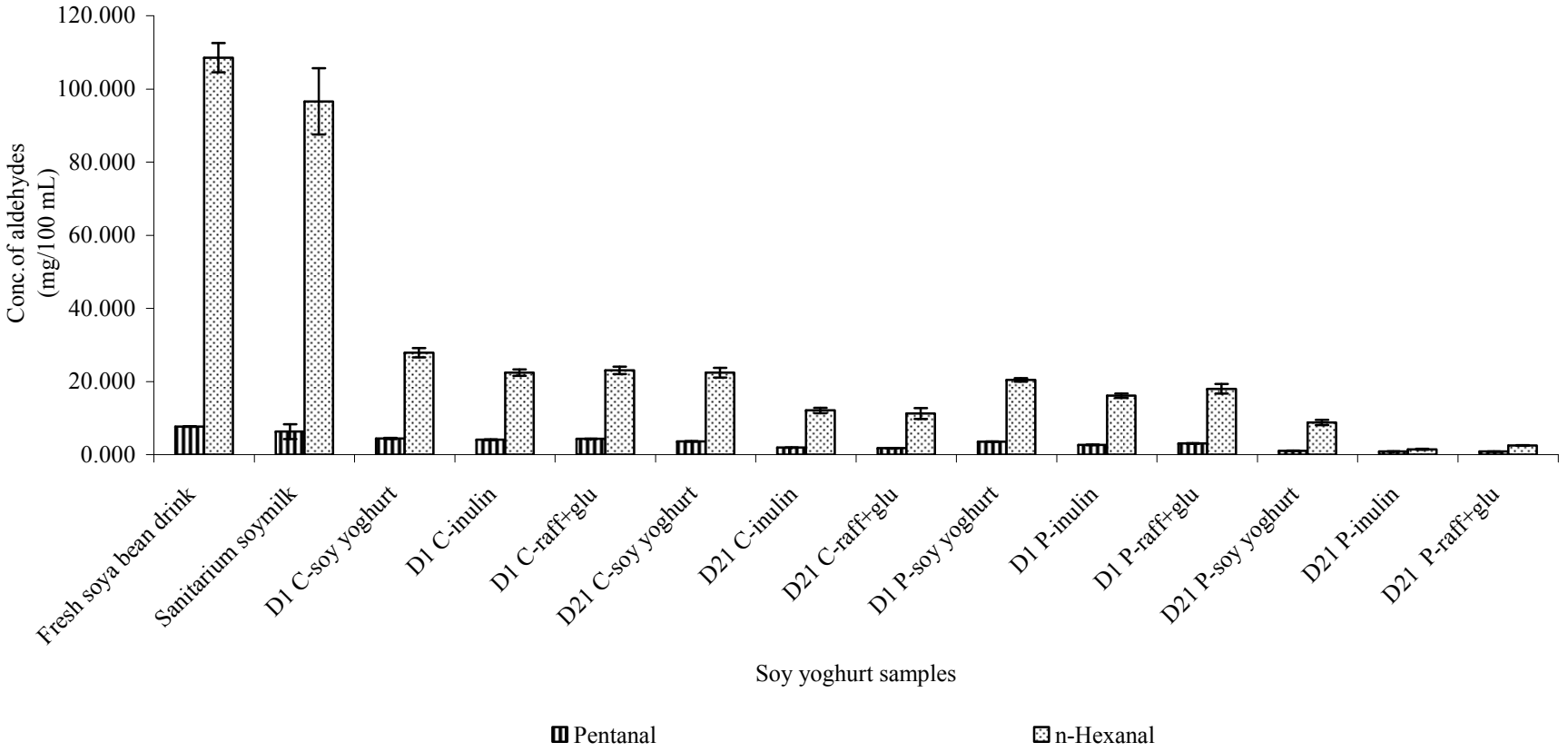

Figure 10.2 Concentration of *n*-hexanal and pentanal in fresh soymilk and soy yoghurt supplemented with or without inulin or raffinose and glucose (raff + glu) fermented with yoghurt culture or yoghurt culture and probiotic organisms; D1 = day 1; D21 = day 21; C = control soy yoghurt containing yoghurt culture (*S. thermophilus* St1342 and *L. delbrueckii* ssp. *bulgaricus* Lb 1466); P = probiotic soy yoghurt containing yoghurt culture, *L. acidophilus* L10, *B. animalis* ssp *lactis* B94 and *L. casei* L26; Error bars represent standard deviation.

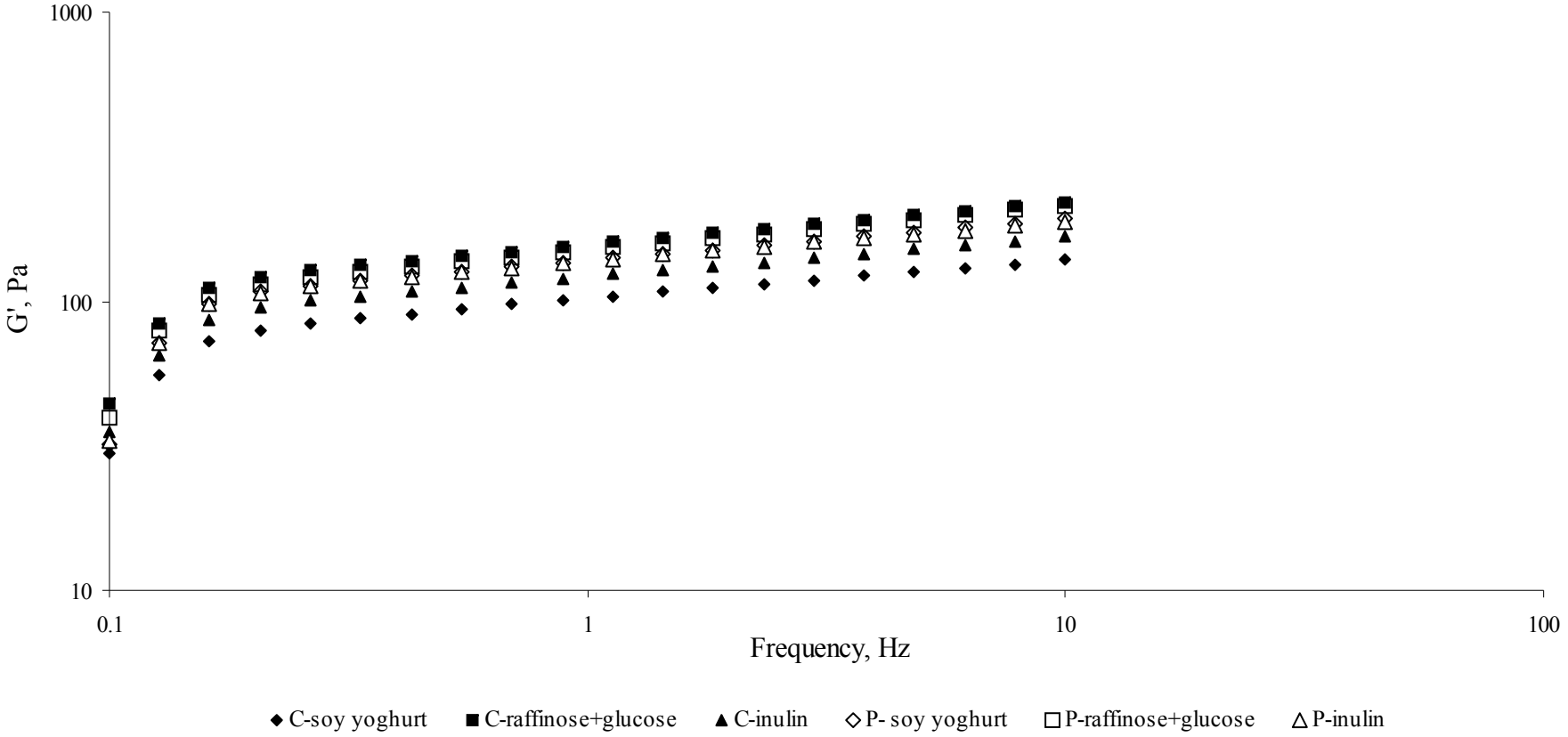

Figure 10.3 Effect of supplementation of soy yoghurt with inulin or raffinose and glucose on the storage modulus (*G'*) compared to soy yoghurt without any supplementation, after 10 d storage at 4°C; C = control soy yoghurt containing yoghurt culture (*S. thermophilus* St1342 and *L. delbrueckii* ssp. *bulgaricus* Lb 1466); P = probiotic soy yoghurt containing yoghurt culture and probiotic organisms (*L. acidophilus* L10, *B. animalis* ssp *lactis* B94 and *L. casei* L26).

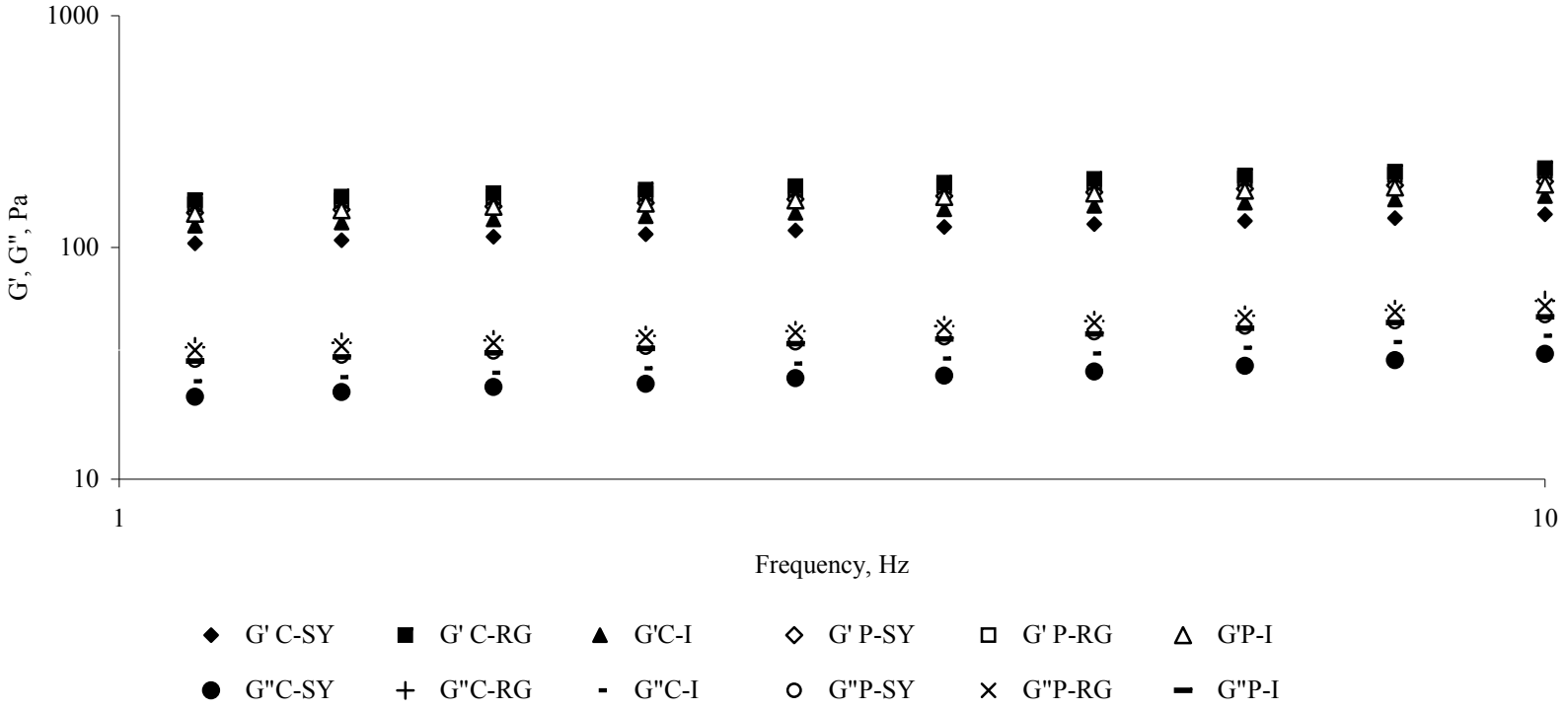

Figure 10.4 Frequency sweeps of soy yoghurt (SY) batches with or without inulin (I), or raffinose and glucose (RG) after 10 d storage at 4^{o}C; C = control soy yoghurt containing yoghurt culture (*S. thermophilus* St1342 and *L. delbrueckii* ssp. *bulgaricus* Lb 1466); P = probiotic soy yoghurt containing yoghurt culture and probiotic organisms (*L. acidophilus* L10, *B. animalis* ssp *lactis* B94 and *L. casei* L26); *G'* = Storage and *G"* = loss modulus.)

11.0 Overall Conclusions and Future Research Direction

11.1 Overall Conclusions

Different termination pH appeared to have no effect on the viability of probiotic organisms in yoghurt and soy yoghurt; however, the survival of probiotic organisms was strain dependant in yoghurt, but not in soy yoghurt. Probiotic organisms thrived well in soy yoghurt; but the growth in yoghurt was variable with *L. acidophilus* L10 showing better growth compared to *B. lactis* B94 and *L. casei* L26. The use of *S. thermophilus* St1342, *L. delbrueckii* ssp. *bulgaricus* Lb 1466 in conjunction with probiotic organisms resulted in an appreciable proteolytic activity which improved the growth of the microorganisms in yoghurt and soy yoghurt during storage at 4^{o}C for 28 days.

In soy yoghurt, the enhanced proteolytic activity by probiotic organisms as adjunct culture improved the production of ACE inhibitory compounds which exerted appreciable *in vitro* ACE inhibitory activity. The development of soy yoghurt containing higher concentration of released bioactive ACE inhibitors and viable probiotics may provide health benefits from these functional compounds more efficiently.

Improvement in stability of probiotic organisms (*L. acidophilus* L10 and *L. casei* L26) in yoghurt during cold storage was achieved by supplementation with prebiotics. Inulin was a better growth promoter of probiotic bacteria than hi-maize. In addition, lower concentrations of inulin were found to be sufficient to stimulate the growth and retain the viability of the selected probiotic organisms and *S. thermophilus* St1342, *L. delbrueckii* ssp. *bulgaricus* Lb 1466 in yoghurt in comparison to hi-maize. Furthermore, addition of inulin and hi-maize to yoghurt changed its textural property by increasing viscosity in comparison to the control.

S. thermophilus St1342, *L. delbrueckii* ssp. *bulgaricus* Lb 1466, *L. acidophilus* L10, *B. lactis* B94, *L. casei* L26, *L. acidophilus* La 4962, *B. longum* Bl 536 and *L. casei* Lc 279 possessed proteolytic enzymes including proteinases, peptidases and aminopeptidases at extracellular and intracellular levels which showed specific activities toward certain substrates. The strains exhibited X-prolyl-dipeptidyl aminopeptidase activity cleaving proline-containing peptides. The proteolytic systems including X-prolyl-dipeptidyl aminopeptidase activity of the bacterial strains in RSM resulted in the release of free amino groups and peptides which substantially improved growth and maintained viability in the RSM. In addition to their proteolytic activity, the selected strains produced a range of bioactive peptides with varying

degrees of ACE-inhibition. *B. longum* Bl 536 and *L. acidophilus* L10 produced the highest activity IC_{50} values of 0.196 and 0.151 mg/mL, respectively. ACE-I was strain and time dependent.

The selected microorganisms exhibited variable α-galactosidase activity with *B. lactis* B94 showing the highest activity in supplemented MRS. The ability to metabolize oligosaccharides during fermentation in soymilk due to the presence of α-galactosidase improved the growth of all organisms resulting in the desired therapeutic level (10^8 cfu/mL). *B. lactis* B94, *S. thermophilus* St1342 and *L. acidophilus* La 4962 reduced raffinose in soymilk by 77.4, 64.5 and 55.9%, respectively.

Bovine yoghurt produced by probiotic bacteria as adjunct culture exerted appreciable *in vitro* ACE-I activity. The enhanced proteolytic activity likely contributed to the observed inhibition and several potential ACE-I peptides were identified from casein and were found to be active inhibitors. In total 8 ACE-I peptides were characterized originating from α_{s2}-CN, κ-CN and β-CN which showed potent inhibitory activities of IC_{50} values ranging from 1.56 to 12.41 μg/mL. These include the two well-known ACE-inhibiting peptides, namely VPP and IPP, which are already identified in many commercial products. However, further studies are necessary to elucidate the formation and degradation mechanism of these bioactive peptides.

β-Glucosidase activity depended on cell growth, strain as well as on the stage of incubation. Since the production of organic acids in soymilk was low during fermentation with each microorganism (*L. acidophilus* L10, *B. lactis* B94 and *L. casei* L26), bacterial growth was substantially high. As a consequence, enzyme activity was at maximum and produced high concentrations of isoflavone aglycones between 63 to 78%, up from 10% in unfermented soymilk at 0 h. The equilibrium point of aglycone and β-glucoside concentrations during fermentation in soymilk may indicate the hydrolytic potential of each microorganism in improving the bioactivity of soymilk.

Metabolic activities of probiotic organisms in soy yoghurt impacted on sensory and rheological characteristics. The addition of inulin, raffinose or glucose to soy yoghurt effectively overcame the low acid production frequently faced in soy-based yoghurt and promoted bacterial cell growth. Furthermore, the probiotic bacteria metabolized aldehydes that are responsible for beany flavour and produced low concentrations of acetic acid which gave a vinegary flavour. The appropriate carbohydrate concentrations in soymilk can improve the

texture quality as well as sensory characteristics without sacrificing flavour quality. Overall the control soy yoghurt, based on the acceptability mean scores, appeared to be more acceptable by the consumer panel than probiotic soy yoghurt. However, in terms of mouthfeel, the supplemented products either with inulin or raffinose/glucose in control and probiotic soy yoghurts were acceptable. Rheological tests have shown that all soy yoghurts, irrespective of supplementation, exhibited solid-like gel characteristics but supplementation with raffinose/glucose produced firmer products. However, the probiotic soy yoghurts showed more viscous and pseudo-plastic properties than the control soy yoghurts. The overall visual appearance, and rheological properties are important physical attributes, and contributed to the overall sensory perception and functionality of these products.

11.2 Future Research Direction

Our results showed that the probiotic organisms (*Lactobacillus* and *Bifidobacterium*) used in this research could release bioactive compounds (peptides and isoflavones) during fermentation in bovine milk and soymilk. Future technological prospects that should be considered or addressed in view of the functionality of probiotic micro-organisms include: (i) fermentation and drying technologies, and (ii) microencapsulation. Furthermore, the probiotic dairy and soy industries should work closely not only with the regulatory authorities but also with the medical profession in order to substantiate the health claims associated with these beneficial micro-organisms. Overcoming these will help ensure the acceptability of probiotic dairy and soy products by the consumer and, hence, safeguard the future of the industry.

Most recently, a number of probiotic strains have been identified that are capable of producing different peptides with varying ACE-inhibitory activity in yoghurt and soy based yoghurt. While the observed activity was strictly strain dependant, it fluctuated with the storage time, which raised an important question of the stability of these peptides during the prolonged storage. The mechanisms to monitor and maintain the stability of these bioactive peptides needs to be assessed to optimize the nutritional and health effect of these compounds. In addition, more work is needed to develop a large-scale fractionation of protein hydrolysates to obtain products enriched with biologically active peptides of specific function that could be used as nutraceutical additives in functional foods.

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Appendix