THE PROTEOLYTIC ACTIVITY OF YOGHURT AND PROBIOTIC BACTERIA FOR THE IMPROVED VIABILITY OF PROBIOTIC BACTERIA IN FERMENTED MILK PRODUCTS

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By

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DEDICATED TO MY FATHER,

LOVING MOTHER AND BROTHER

ABSTRACT

The proteolytic activity of various strains of yoghurt and probiotic bacteria, particularly their amino-, di-, tri- and endo-peptidase activities was studied. Nine strains of *Streptococcus thermophilus*, 6 strains of *Lactobacillus delbrueckii* ssp. *bulgaricus*, 14 strains of *Lactobacillus acidophilus* and 13 strains of *Bifidobacterium* ssp. were screened for proteolytic activity by using the o-phthaldialdehyde based spectrophotometric assay. Those strains showing the highest and lowest proteolytic activity were further studied for their peptidase activities at the extracellular and intracellular levels. Aminopeptidase activities were measured using chromogenic substrates (p-nitroanilide derivatives of L-anomers of leucine, lysine, alanine, proline, arginine, and methionine). Dipeptidase activities were measured using dipeptides including Ala-Met, Leu-Tyr, Leu-Gly, Ala-His, and Pro-Ile as substrates. Endopeptidase and tripeptidase activities were detected using thin-layer chromatography.

The amounts of free amino groups released by *S. thermophilus*, *L. delbrueckii* ssp. *bulgaricus* and *L. acidophilus* strains were higher than those by *Bifidobacterium* strains. Aminopeptidase activity was detected for all bacterial strains both at the extracellular and intracellular levels. The specific activity towards the six substrates studied was higher at the intracellular level for all strains. High dipeptidase activity was also demonstrated by all bacterial strains for the substrates tested with greater specificity at the intracellular level for *L. delbrueckii* ssp. *bulgaricus*, *L. acidophilus*, and *Bifidobacterium* ssp., whereas *S. thermophilus* had greater dipeptidase activity at the extracellular level. All bacterial cultures tested were able to hydrolyse large biologically active peptides, bradykinin, Ala-Ala-Ala-Ala-Ala and the tripeptide substrate Gly-Ala-Tyr at both the extracellular and intracellular levels. The same can be deduced for the tripeptide substrate Gly-Ala-Tyr. However, with the substrates ending with a C-terminal of phenylalanine, the hydrolysis only occurred at the intracellular level.

The nature of the relationship between yoghurt bacteria (*S. thermophilus* 2002, *S. thermophilus* 2014, *L. delbrueckii* ssp. *bulgaricus* 2501 and *L. delbrueckii* ssp. *bulgaricus*

2515) and probiotic bacteria (*L. acidophilus* 2405, *L. acidophilus* 2415, *B. thermophilum* 20210, *B. pseudolongum* 20099, and *B. longum* 1941) when in co-culture was investigated. Viable counts of yoghurt and probiotic bacteria were obtained by pour plating appropriate dilutions on appropriate media and percentages of inhibition were also calculated. A total of nine strains of yoghurt bacteria and probiotic bacteria were used in this study. *S. thermophilus* 2002 and both *L. delbrueckii* ssp. *bulgaricus* 2501 and 2515 strains showed strong inhibition which could be due to bacteriocin like inhibitory substance (BLIS) produced by both *L. acidophilus* strains. *Bifidobacterium* ssp. was not inhibited in this study. *S. thermophilus* 2002, *L. delbrueckii* ssp. *bulgaricus* 2501, and *L. acidophilus* 2415 strain were inhibited by *L. acidophilus* 2405 strain. These inhibitions were observed during initial screening with modified spot on lawn technique, but not observed with the agar well assay technique.

Casein degradation by selected strains of yoghurt and probiotic bacteria was evaluated by SDS-PAGE electrophoresis at different incubation times with bacterial suspensions obtained from cultures grown in skim milk at 37°C. All casein fractions incubated with the intracellular samples were digested at a faster rate than that in cell wall extracts. Analysis of protein bands in the SDS-PAGE bands via density provided better quantification of degradation products than that estimated by visual observation of the gels. This was done by capturing an image of the gels via a Luminescent Image Analyser and the data was analysed with Image Gauge Software.

Most bacterial strains showed 0% hydrolysis of α-casein, β-casein, and κ-casein at the cell wall level when compared to that at the intracellular level. *S. thermophilus* 2002 degraded α-casein and β-casein at the fastest rate when compared to other single bacterial cultures studied. Similar results were observed with *S. thermophilus* 2014 except for its action towards κ-casein showing a preference for hydrolysis at the intracellular level. Both *L. delbrueckii* ssp. *bulgaricus* 2501 and 2515 readily attacked all casein fractions, particularly at the intracellular level. Most of the hydrolysis occurred after 4 hours of incubation. *L. acidophilus* (strains 2405 and 2415) also attacked the casein fractions at the intracellular level, where 64%, 70%, and 82% hydrolysis of α-, β-, and κ-casein,

occurred, respectively. On the other hand, *B. thermophilum* 20210, and *B. infantis* 1912 did show caseinolytic activity, but a slightly different pattern to that observed with the other bacterial strains tested. *B. infantis* 1912 appeared to show casein hydrolysis at both cell levels; however the rate of hydrolysis was slower than that of the other probiotic strain and yoghurt strains. This agrees with earlier findings in this study, where *Bifidobacterium* ssp. are slow growing organisms in milk when compared with the other bacteria cultures. *B. thermophilum* 20210 grew faster than that of *B. infantis* 1912 where hydrolysis of the casein fractions started as early as after 2 hours of incubation.

The RP-HPLC method used was chosen so that separation and quantification of the six major bovine milk proteins was possible at the same time. The bovine casein group (α_s, β, and κ-casein) and the whey proteins (β-lactoglobulin and α-lactalbumin) were separated and quantified using reverse-phase HPLC. Bovine milk proteins were quantitatively determined by using standard curves, which were developed by measuring peak areas at various known amounts of injected milk proteins. *S. thermophilus* 2014 and *L. acidophilus* 2405 showed a preference for α-casein degradation when compared to the other bacterial single cultures studied. On the other hand, *Bifidobacterium* ssp. (1941 and 20099) appeared to hydrolyse α-casein at a slower rate. *B. thermophilum* (20210) appeared to break the α_s-casein fraction into a smaller peptide, which indicates that it actively degraded the casein fraction at a faster rate than the other *Bifidobacterium* ssp. strains, studied, thus the smaller peak size.

When the mixed bacterial cultures were studied for α-casein hydrolysis, *S. thermophilus* 2002 + mix, *S. thermophilus* 2014 + mix, and *L. acidophilus* 2415 + *Bifidobacterium thermophilum* 20210, showed significant hydrolysis ($P<0.017$). It appears that the combination of yoghurt bacteria in a mixed culture did degrade α-casein at a faster rate than when cultured alone as would have been expected, indicating they actively break down existing bonds and form smaller peptide fractions. There was not much change in α_s-casein degradation patterns during the 12-hour incubation period by the starter culture strains studied. A similar trend to that of α-casein hydrolysis was observed with β-casein degradation patterns.

L. acidophilus 2405 actively hydrolysed β-casein, breaking down existing bonds and forming new smaller peptides. Similar observations were also seen with the yoghurt bacteria, *S. thermophilus* 2002 and *S. thermophilus* 2014. The other yoghurt bacteria, *L. delbrueckii* ssp. *bulgaricus* 2501 and 2515 did not appear to degrade β-casein at the same rate as that exhibited by *S. thermophilus* strains. The peak corresponding to β-casein increased in size throughout the 12 hour incubation period in the mixed culture of *S. thermophilus* 2002 + *L. delbrueckii* ssp. *bulgaricus* 2501. This could be explained in terms of the highly proteolytic nature of both of *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* and their symbiotic relationship, where the covalent bonds within the protein fraction are broken down to form new amino acids and may form links with other peptides, thus forming a larger peptide fraction.

Yoghurt starter culture strains, T-1 ST, Y-1 ST, T-4 ST, and Y-2 LB, appeared to actively degrade the β-casein fraction over time, where the peak corresponding to β-casein decreased in size during the 12 hour incubation period. Probiotic starter culture T-1 LA was also shown to actively degrade β-casein, where there was a significant ($P<0.034$) decrease in β-casein peak size between 6 and 12 hours. On the other hand, there was a significant decrease ($P<0.006$) in κ-casein peak size observed with *L. delbrueckii* ssp. *bulgaricus* 2501, which means that *L. delbrueckii* ssp. *bulgaricus* 2501 was able to break down peptide bonds within the κ-casein fraction of milk proteins. It was observed that all mixed bacterial cultures studied degraded κ-casein to some extent, when compared to that of κ-casein in the control milk (7.18 mg/ml); however, this was not significantly different ($P<0.456$).

When bacterial cultures were studied for degradation of α-lactalbumin, it was observed that the heights of α- lactalbumin peaks were reduced and several additional peaks appeared with migration times closer to those of the main whey protein (α-lactalbumin). There was no particular trend observed for α-lactalbumin utilisation by the mixed bacterial cultures. It appeared that the mixed bacterial cultures hydrolysed the α-lactalbumin protein, however it was not significantly different ($P>0.576$). No particular trends were observed with respect to degradation of β-lactoglobulin-A, as was exhibited

by the bacterial combinations. There were no changes in β-lactoglobulin A peak size observed during the 12 hour incubation, except for a slight decrease observed in the bacterial combinations of *L. acidophilus* 2405 + *B. longum* 1941 and that of *S. thermophilus* 2014 + *L. delbrueckii* ssp. *bulgaricus* 2515, but it was not significant enough ($P>0.480$) to exclude the possibility that the difference is due to random sampling variability. *B. thermophilum* 20210 also showed a preference for the B fraction of β-lactoglobulin, where there was a significant decrease in peak size observed during the first 4 hours of incubation when compared to that of the control milk. *L. delbrueckii* ssp. *bulgaricus* 2501 was observed to be the most active towards utilising β-lactoglobulin B when compared to the other yoghurt and probiotic bacteria studied. The rest of the mixed combinations appeared to either grow at a slower rate or did not need any nutrients at that stage of their growth, where β-lactoglobulin B peak size remained unchanged.

The influence of fortifying milk with some amino acids on growth and acid production by *S. thermophilus* 2002, *L. delbrueckii* ssp. *bulgaricus* 2501, *L. acidophilus* 2405, and *Bifidobacterium* ssp. 20210 was studied. Total amino acids quantified in the bacterial cultures were less than that of the control RSM, where the lowest was observed for *L. delbrueckii* ssp. *bulgaricus* 2501 and *L. acidophilus* 2405 (39.8 and 43.2 μg/ml respectively). *S. thermophilus* 2002 and *B. thermophilum* 20210 released the highest amounts of tyrosine which indicates a high degree of proteolysis. Both *S. thermophilus* strains (2002 and 2014) had utilized the amino acids aspartic acid, threonine, serine, glutamic acid, alanine, valine, leucine, and lysine.

The rest of the amino acids tested were not utilized to a great extent. *L. delbrueckii* ssp. *bulgaricus* 2501 appeared to utilize amino acids to a greater extent than that of *L. delbrueckii* ssp. *bulgaricus* 2515. Total amino acids quantified in *L. delbrueckii* ssp. *bulgaricus* 2515 filtrates (96.4 μg/ml) was much greater than that of *L. delbrueckii* ssp. *bulgaricus* 2501 (39.8 μg/ml). *L. acidophilus* 2415 released a greater quantity of amino acids than *L. acidophilus* 2405, 137.9 μg/ml and 39.8 μg/ml, respectively. *L. acidophilus* 2415 had a preference for the utilization for most of the amino acids except for tyrosine and cystine and cysteine. On the other hand, *B. thermophilum* 20210 appeared to utilize a

greater amount of amino acids than that of *B. longum* 1941. When compared to control RSM, *B. thermophilum* 20210 released about 69.9 μg/ml of the total amino acids available compared to 114.6 μg/ml for *B. longum* 1941. Bacterial cultures were also grown in the presence of amino acids, in particular, those containing sulphur, e.g. cysteine and methionine to assess their influence on cell growth and acid production. Bacterial cultures of *S. thermophilus* 2002, *L. delbrueckii* ssp. *bulgaricus* 2501, *L. acidophilus* 2405, and *B. thermophilum* 20210, were grown alone in single cultures and in co-culture with each other. Control cultures were not supplemented with amino acids. The differences in the log cell counts among the yoghurt bacteria when supplemented with the amino acids was lower when compared to the control group (not supplemented with amino acids).

The preference for the amino acids tested varied with each strain. On the other hand, the effect of the supplementation of various amino acids on the bacterial cell counts of the probiotic bacteria, *L. acidophilus* 2405 and *B. thermophilum* 20210 was notably different to that of the yoghurt bacteria studied. The log viable counts of *B. thermophilum* 20210, when co-cultured with *S. thermophilus* 2002, *L. delbrueckii* ssp. *bulgaricus* 2501, and *L. acidophilus* 2405 was significantly influenced ($P>0.20$) by the various amino acid supplementations. The results showed that *B. thermophilum* 20210 had a preference for the di-sulfide containing amino acids. The results of these studies showed that the probiotic bacteria had a preference for di-sulfide containing amino acids whereas the yoghurt bacteria had a preference for the other amino acids tested.

The effects of addition of proteolytic strains of *Lactobacillus delbrueckii* subsp. *bulgaricus* to commercial ABT starter cultures on the texture of yoghurt, exopolysaccharide production and survival of starter bacteria were studied. The firmness of set yoghurts was measured with a cone-penetrometer and the apparent viscosity with a Brookfield viscometer. EPS was extracted by precipitation with ethanol. Supplementation of proteolytic strain of *L. delbrueckii* subsp. *bulgaricus* 2501 or 2515 to ABT-1 or ABT-4 starter culture reduced the fermentation time in making yoghurt. Yoghurts made with ABT-1 and ABT-4 with added *L. delbrueckii* ssp. *bulgaricus* 2501

fermented in the shortest time of 5 h and 33 min and 5 h and 8 min, respectively. Quantities of EPS extracted from the different yoghurt batches did not show any significant differences ($P<0.222$). In general, the firmness improved with the addition of the proteolytic strains of *L. delbrueckii* subsp. *bulgaricus*. Addition of *L. delbrueckii* subsp. *bulgaricus* to ABT-1 and ABT-4 did not produce significant ($P<0.001$) improvement in firmness of yoghurt compared with the mix starter batches (composed of different bacterial strains). Significant differences ($P<0.001$) were observed in the viscosity of yoghurts supplemented with different combinations of starter cultures. However, the amounts of EPS isolated from yoghurt cultures did not correlate with the viscosities of the yoghurts. Viability of probiotic bacteria improved in the yoghurt product made with mix starter culture with the added *L. delbrueckii* subsp. *bulgaricus* proteolytic strains.

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CERTIFICATE

This is to certify that the thesis entitled "THE PROTEOLYTIC ACTIVITY OF YOGHURT AND PROBIOTIC BACTERIA FOR THE IMPROVED VIABILITY OF PROBIOTIC BACTERIA IN FERMENTED MILK PRODUCTS' submitted by Ms Amal Shihata in partial fulfillment of the requirements for the award of the degree of Doctor of Philosophy in the School of Molecular Sciences at Victoria University is a record of bonafide research work carried out by her under my personal guidance and supervision and the thesis has not previously formed the basis for the award of any degree, diploma or other similar title.

[redacted]

Werribee, Australia
Date: 15. 4. 2002

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Thesis Supervisor

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REFEREED RESEARCH PAPERS

(1) **A.Shihata** and N.P.Shah. 2002. Influence of addition of proteolytic strains of *Lactobacillus delbrueckii* subsp. *bulgaricus* to commercial ABT starter cultures on texture of yoghurt, exopolysaccharide production and survival of bacteria. International Dairy Journal, 12, 765-772. (Presented in Chapter 4 of this thesis).

(2) A.Shihata and N.P.Shah. 2000. Proteolytic profiles of yoghurt and probiotic bacteria. International Dairy Journal, 10; 401-408. (Presented in Chapter 8 of this thesis).

CONFERENCE PRESENTATIONS

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(3) **Shihata, A** and Shah, N.P. 1999. Aminopeptidase, endopeptidase, dipeptidase, and tripeptidase activities of selected yoghurt and probiotic bacteria. A poster presented at the 10^{th} World Food Congress, Sydney, Australia, Australia, October 3-8.

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(5) Shihata, A and **Shah, N.P**. 2000. Casein and whey protein degradation patterns by selected lactic acid bacteria. A paper presented at the American Dairy Science Association Annual conference, Baltimore, MD, July 24-28 (J. Dairy Science 83 (supplement):88).

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AB =	*L. acidophilus* and *Bifidobacterium* ssp.
ABT	*L. acidophilus*, *Bifidobacterium* ssp., and *S. thermophilus*
BB	*Bifidobacterium* ssp.
BLIS	Bacteriocin-like inhibitory substance
CE	Capillary electrophoresis
CFE	Cell free extracts
CFU	Colony forming units
CN	Casein
CWE	Cell wall extracts
EE	Cell wall extracellular extract
EPS	Exopolysaccharide
HPLC	High performance liquid chromatography
IE	Intracellular extract
LA	*L. acidophilus*
LAB	Lactic acid bacteria
LB	*L. delbrueckii* ssp. *bulgaricus*
LG	Lactoglobulin
MRS	de Mann Rogosa Sharpe
NNLP	Nalidixic acid, neomycin sulfate, lithium chloride and paromomycin sulfate
OPA	*o*-phthaldialdehyde
RP-HPLC	Reverse-phase high performance liquid chromatography
RSM	Reconstituted skim milk
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	Scanning electron microscopy
ST	*S. thermophilus*
TCA	Trichloroacetic acid
TEMED	N, N, Ń, Ń-tetramethylethylenediamine
TPA	Texture profile analysis

Chapter 1 Introduction

Metchnikof, in his fascinating treatise 'The Prolongation of Life', propounded that the longevity of Bulgarians was in part due to their consumption of large quantities of fermented milks containing lactobacilli. This observation has led to burgeoning activity on the elucidation of the role of lactic cultures and cultured milk products in alleviation of gastrointestinal disorders in humans and animals. Recent advances in our knowledge of the biosynthetic activities of lactic acid bacteria (LAB) and their antagonistic action against pathogenic, toxigenic and putrefactive organisms have added a new dimension to the importance of fermented milks in human nutrition and health (Kailasapathy and Chin, 2000).

Since Metchnikoff's time, several lactobacilli including *L. acidophilus* and *Bifidobacterium bifidum* have been investigated as possible candidate organisms. Metchnikoff believed that life could be prolonged by the ingestion of soured milk containing cells of *Lactobacillus bulgaricus* now known as *L. delbrueckii* ssp. *bulgaricus*. In the 1920's attempts to implant *L. delbrueckii* ssp. *bulgaricus* in the intestine were unsuccessful; however, it was found that *L. acidophilus* would survive and implant. Increasing the number of lactobacilli in the human intestinal tract, by consuming high levels of *L. acidophilus*, was reported by several investigators (Gilliland *et al.*, 1978; Kim, 1988). Beneficial roles of *L. acidophilus* in humans needing therapy for various types of intestinal illnesses have been studied by Kopeloff (1926) and Rettger *et al.* (1935). They mentioned that constipation, diarrhea, and other intestinal disorders could be satisfactorily treated by *L. acidophilus* (Kim, 1988).

There is renewed interest in using LAB as probiotic food additives to enhance immune function and prevent gastrointestinal infection. Unfortunately, many commonly used lactic acid bacterial preparations have been found to lack efficacy. Therefore, research has focused on identifying new lactic acid bacterial strains with health-promoting properties.

Probiotic bacteria (*Lactobacillus acidophilus* and *Bifidobacterium* ssp.) grow slowly in milk because of lack of proteolytic activity, and the usual practice is to add yoghurt bacteria (*Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*) to probiotic products to reduce the fermentation time. *Lactobacillus delbrueckii* ssp. *bulgaricus* produces essential amino acids owing to its proteolytic nature, and the symbiotic relationship of *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus* is well established; the former organism produces amino nitrogen for the latter organism (Shihata & Shah, 2000).

LAB are characterized by their high demand for essential growth factors such as peptides and amino acids. However, milk does not contain sufficient free amino acids and peptides to allow growth of LAB. Therefore, these LAB possess a complex system of proteinases and peptidase, which enable them to use milk casein as a source of amino acids and nitrogen (Shihata & Shah, 2000).

In recent years, fermented milk products containing *L. acidophilus* and *Bifidobacterium* ssp. have been developed. There is an increasing interest in dairy products containing specific bacterial species with potential health benefits. However, the slow growth of *L. acidophilus* and *Bifidobacterium* ssp. poses major difficulties for market expansion of probiotic products. Use of most proteolytic strains of *S. thermophilus*, *L. delbrueckii* ssp. *bulgaricus*, *L. acidophilus* and *Bifidobacterium* ssp. could enhance the growth and viability of probiotic bacteria in products over a storage period.

This study aimed at achieving two major objectives. The first objective was to evaluate the proteolytic ability of various strains of *S. thermophilus*, *L. delbrueckii* ssp. *bulgaricus*, *L. acidophilus* and *Bifidobacterium* ssp. The second major objective was to study the best mix of bacterial strains with the greatest proteolytic capabilities in order to improve the viability of probiotic bacteria in a fermented milk model. The specific objectives of this project were to:

(1) Select and identify the most proteolytic strains of yoghurt bacteria and probiotic bacteria,

(2) Identify the strains of yoghurt bacteria that stimulate or inhibit the growth of probiotic bacteria,

(3) Study the peptidase profile in proteolytic strains of *S. thermophilus*, *L. delbrueckii* ssp. *bulgaricus*, *L. acidophilus* and bifidobacteria,

(4) Study the amino acid profile released by the proteolytic strains of *S. thermophilus*, *L. delbrueckii* ssp. *bulgaricus*, *L. acidophilus* and *Bifidobacterium* ssp.,

(5) Investigate the exact amino acid requirement of *L. acidophilus* and bifidobacteria,

(6) Study the protein degradation patterns of proteolytic strains of yoghurt and probiotic bacteria, and

(7) Study the influence of addition of proteolytic strains of *L. delbrueckii* ssp. *bulgaricus* to commercial ABT starter cultures on texture of yoghurt, exopolysaccharide production and survival of bacteria.

Chapter 2 of this thesis contains a review of the literature, chapter 3 contains materials and methods used and chapter 4 deals with the proteolytic and peptidase activity of yoghurt and probiotic bacteria. Chapter 5 deals with the study of the nature of interaction, associative and/or inhibitory growth relationship that occurs between yoghurt and probiotic bacteria. Protein degradation and utilization patterns of selected proteolytic bacteria are represented in Chapter 6. First part of chapter 7 deals with the study of the amino acid profiles of selected proteolytic bacteria and the second part investigates the effect of supplementation of various amino acids on the viable counts of yoghurt and probiotic bacteria. Chapter 8 deals with the influence of addition of proteolytic strains of *Lactobacillus delbrueckii* ssp. *bulgaricus* to commercial ABT starter cultures on texture of yoghurt, exopolysaccharide production and survival of bacteria. Chapter 9 presents a summary of the research findings and a final conclusion to the study with proposed future research direction. A list of references used is presented in Chapter 10.

Chapter 2 Literature Review

2.1 Yoghurt & fermented milks

Since the 1960s, there has been a worldwide increase in the industrial production of fermented milks, especially yoghurt. Yoghurt has been part of the diet in Southeastern Europe and the Middle East for millennia and is now part of the dairy counters even in the small grocery stores in many countries. It is a cultured milk product easy to make with the help of LAB. Compared to other milk products such as cheese, ice cream, or butter, yoghurt contains most milk constituents, except lactose, most of which is converted into lactic acid by starter bacteria. This acid gives the yoghurt a pleasant acidic flavour and, at the same time, the sweetness caused by lactose is reduced. The word "yoghurt" is derived from the Turkish word 'jugurt' (Tamime & Deeth, 1980). Several factors account for the success of yoghurt: its natural image, its organoleptic characteristics (fresh and acidulated taste and characteristic flavour), nutritional, prophylactic and therapeutic properties, and its moderate cost (due to the high production lines) (Birollo *et al.*, 2000).

The consistency, flavour and aroma vary from one district to another. In some areas yoghurt is produced in the form of a highly viscous liquid, whereas in other countries it is produced in the form of a softer gel. Yoghurt is also produced in frozen form as a dessert, or as a drink. The flavour and aroma of yoghurt differ from those of other acidified products, and the volatile aromatic substances include small quantities of acetic acid and acetaldehyde (Teknotext AB, 1995).

Yoghurt is typically classified as follows:

- Set type incubated and cooled in the package.
- Stirred type incubated in tanks and cooled before packing.
- Drinking type similar to stirred type, but the coagulum is "broken" to a liquid before being packed.
- Frozen type incubated in tanks and frozen like ice cream.

- Concentrated incubated in tanks, concentrated and cooled before being packed. This type is sometimes called strained yoghurt, labneh or labaneh (Teknotext AB, 1995).

2.1.1 Definition of yoghurt and fermented milks

The definition of "yoghurt" according to the Codex Alimentarius (Std n°A-11a/1975) is "a coagulated milk product obtained by specific lactic acid fermentation, through the action of *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus*, from cow's milk with or without addition of powdered milk or powdered skim milk. The microorganisms in the final product must be viable and abundant". The use of lactic acid bacteria other than the 2 species cited in the definition is not permitted in most European countries. In this case, yoghurt containing *Bifidobacterium* ssp. or *Lactobacillus acidophilus* would be classified as a "fermented milk". In other countries, such as the UK, Canada and the USA, the addition of other lactic acid bacteria to the two species used to make yoghurt is permitted (Gilliland, 1991). According to the Australian Food Standards Code (Standard H8), yoghurt must have a pH of ≤ 4.5 and must be prepared with *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* or other suitable lactic acid bacteria (Shah, 2000).

Yoghurts have been a category of considerable growth for the dairy industry over the past decade. Yoghurt has a favourable consumer image as a healthy snack. Reflecting this, low-fat and diet variants account for more than half the annual supermarket sales of yoghurt. The segment is dominated by strong international brands such as Ski, Yoplait and Nestle. Growth in yoghurt has been underpinned by continued product innovation in the areas of packaging, flavours and the use of probiotic cultures, as well as new products such as drinking yoghurts (Dairy Australia, 2003).

2.1.2 Milk fermentation

To ferment milk, concentrated cultures of bacteria are inoculated into pasteurized milk which has been enriched with milk solids and is then incubated at 40-44°C for 4 to 5 hours. During the fermentation, lactic acid produced from lactose by the yoghurt bacteria, whose population increases 100- to 10,000- fold to a final concentration of

approximately 10^9/mL. The reduction in pH, due to the production of lactic acid, causes a destabilization of the micellar casein at a pH of 5.1-5.2 and with a complete coagulation occurring around pH 4.6. The caseins do not precipitate but form a gel. Its ability to retain all the water present in the milk is the result of a peculiar microstructure of the protein network. It consists of short branched chains of micelles and resembles a sponge with very small pores. At the desired final pH, the coagulated milk is cooled quickly to 4-10°C to slow down the fermentation (Gilliland, 1991).

Yoghurt is unique from both the structural as well as compositional viewpoints, because it is solid and has the highest water content of all solid milk products. Yoghurt that has been stored for a long period of time may show some syneresis which is the separation of a liquid phase from the gel. This is only a minor cosmetic defect and the liquid soaks back into the body of the yoghurt as soon as the yoghurt is stirred. Electron microscopy reveals interesting features in the development of yoghurt structure. The crucial condition in yoghurt making is the heating of the milk. Its temperature must reach at least 85°C (90°C is more commonly used) and held at this temperature for at least 30 min. This treatment alters the casein micelles and prepares them for the unique structure to form (Webb *et al.*, 1978).

Casein micelles are described as protein globules about 100 nm in diameter and consist of yet smaller submicelles. Of several different casein molecules, kappa-casein (κ-casein) on the surface of the micelles has a pivotal role in their stability. As long as this protein is intact, the micelles stay in milk as individual entities. Any change in the integrity of κ-casein destabilizes the micelles leading to aggregation. Casein micelles in unheated milk have relatively smooth surfaces with very small humps caused by the submicelles. Heating above 85°C leads to an interaction between β-lactoglobulin (one of the whey proteins) and κ-casein on the casein micelle surface. The result is a complex which makes the casein micelle surface markedly coarser. Casein micelles with the κ-casein-β-lactoglobulin complex formed on their surfaces have a limited ability to aggregate (Webb *et al.*, 1978).

2.2 Probiotics

Probiotics are non-pathogenic, host-derived microorganisms, which beneficially affect the host by improving microbial balance of the target niche and have been advocated to restore and maintain health for over one hundred years. This concept has received increased attention for many reasons including elevated antibiotic resistance, consumer demands for natural health remedies and progress in science producing compelling evidence in favour of probiotic organisms (Reid, 2000).

The concept of ingesting live microorganisms for the purpose of improving one's intestinal health and general well-being can be traced back to the beginning of the 20th century. This practice is now referred to as 'probiotics' and is the subject of intense scientific research directed toward obtaining effective probiotic bacteria and establishing their health benefits. Although numerous genera of bacteria (and yeasts) are currently being marketed as probiotic cultures throughout the world, the two most commonly used genera are Lactobacillus and *Bifidobacterium*. The market for probiotic cultures is very significant is Asia, particularly Japan, and has attained significance in Europe during the past decade. In recent years, there has been a growing body of scientific evidence bolstering specific health claims of certain probiotic strains. The health claims are quite diverse, ranging from prevention of diarrhea to reduction in cholesterol, highlighting the tremendous potential for the probiotic field (O'Sullivan, 2001).

The emergence of antibiotic-resistant bacteria and natural ways of suppressing the growth of pathogens has contributed to the concept of 'probiotics'. Probiotic bacteria not only compete and suppress 'unhealthy fermentation' in human intestine, but also produce a number of beneficial health effects of their own.

2.2.1 Definition

The word 'probiotic', from the Greek 'for life', has over the past few years been used in several different ways (Gomes and Malcata, 1999). Later, the term probiotic was introduced by Lilly and Stillwell (1965) to describe substances produced by one microorganism that stimulated the growth of other microorganisms. Since then,

probiotics have been defined in many different ways, depending on our understanding of the mechanisms of their effects on the health and well being of humans (Salminen *et al.*, 1998).

The most common definition currently used is that of Fuller (1989) who defines probiotics as 'Live microbial feed supplements which beneficially affect the host animal by improving its intestinal microbial balance'. The definition of probiotic was broadened in the current decade by Havenaar and Huis in't Veld (1992). Presently, probiotics are defined as "viable microorganisms (lactic acid and other bacteria, or yeasts applied as dried cells or in a fermented product) that exhibit a beneficial effect on the health of the host upon ingestion by improving the properties of its indigenous microflora". Recently a European expert group proposed a definition that also included mechanisms not mediated by change in microflora (Salminen *et al.*, 1998).

Probiotics have been consumed in foods such as yoghurt for perhaps thousands of years, and while the 'cultures' were thought to have beneficial effects, it was not until the 1900s that scientists began to investigate the reasons for those benefits (Anon, 1996).

2.2.2 *Selection criteria for probiotics*

There is increasing evidence that probiotics can benefit the human host by acting as a first line of defence against disease-causing pathogens by improving the intestinal microflora. However, to have functional probiotic strains with predictable and measurable health benefits, a concerted effort for strain selection is required. There is no agreed set of selection criteria for classifying a viable bacterial strain as a probiotic. Common criteria used for isolating and defining probiotic bacteria and specific strains include the following: genera of human origin, stability against bile, acid, enzyme and oxygen, ability to adhere to intestinal mucosa, colonization potential in the human gastrointestinal tract, production of antimicrobial substances, and demonstrable efficacy and safety.

The parameters for screening micro-organisms for potential valuable probiotic strains should include the fact that there is a necessity for the strain to be viable and

metabolically active within the gastrointestinal tract and biologically active against the identified target. In addition, it is important that viability of the strain and stability of the desirable characteristics of the strain can be maintained during commercial production as well as in the final product. A combination of *in vitro* studies, including clinical trials, is required.

Most current probiotics have been selected using these criteria. However, in some cases the outcome of such selection has been questioned, especially the requirement for organisms of human origin. The most often used genera are *Lactobacillus* and *Bifidobacterium*. Probiotic bacteria with desirable properties and documented clinical effects, include *L. acidophilus* (NCFB1478), *L. casei* Shirota strain, *Lactobacillus* GG (ATCC53103), *B. animalis* Bb12, *L. johnsonii* LJ1 and *L. reuteri* (Iwana *et al.*, 1993).

Several studies have been reported using each one of these probiotics, documenting their health benefits and specific effects. These strains have also been further studied for efficiency of curing various intestinal dysfunctions and they may offer alternate methods for dietary treatment of intestinal disorders. A potentially successful probiotic strain is expected to have several desirable properties in order to be able to exert its beneficial effects. The selection criteria that have been considered to be relevant for any potential probiotic microorganism are listed in Table 2.1.

Table 2.1 Selection criteria for probiotic microorganisms[a]

Probiotic strains properties	Remarks
Human origin, if intended for human use	May be important for species-dependent health effects.
Acid and bile stability	Important for oral administration, may not be relevant for other applications; for survival through the intestine, retaining cell integrity, maintaining adhesiveness and metabolic activity.
Adhesion to mucosal surfaces	Immune modulation, competitive exclusion of pathogens, prevention of pathogen adhesion.
Safe for food and clinical use	Accurate strain identification and characterization. Documented safety. No degradation of intestinal mucus. No invasion.
Clinically validated and documented health effects	Dose-response data for minimum effective dosage for each particular strain and in different products.
Good technological properties	Strain stability, phage resistance, survival in product (if viable organisms are required), culturable at large scale, no negative effect on product flavour, oxygen resistance.

Source: Ouwehand *et al.* (1999)

Most commonly, *L. acidophilus*, *L. casei*, *Bifidobacterium bifidum* and *B. longum* have been used as probiotics in humans (Playne, 1994). Probiotic bacteria could be applied to balance disturbed intestinal microflora and related dysfunction of the gastrointestinal tract (Kailasapathy and Chin, 2000).

The use of *Bifidobacterium* ssp. and/or *L. acidophilus* in fermented or culture-containing milks became popular by the end of the 1970s as a result of the tremendous increase in knowledge encompassing the taxonomy and ecology of bifidobacteria. Their popularity has further increased owing to their reduced acidification during post-processing storage and their relatively high yield of L(+)-lactic acid compared with D(-)-lactic acid. In recent years, much work on bifidobacteria, regarded as microorganisms targeted for technological and therapeutic applications, was performed in Japan, but other countries (e.g. Denmark, Germany, Poland, Russia, UK and USA) also became involved (Gomes and Malcata, 1999).

2.2.3 Lactic acid bacteria

LAB have been used to ferment foods for at least 4000 years. Without understanding the scientific basis, people used foods containing LAB to produce cultured foods with improved preservation and with different characteristic flavors and textures from the original food. LAB are widely used as starter cultures in the production of food and feed. Fermentation of milk, vegetables, sausages, beverages and bakery products results in products with changed composition and taste, and with prolonged shelf life. LAB are also used as silage inoculates to enhance the fermentation and reduce the risk of spoilage. LAB are used for many fermented milk products from all over the world as well, including yoghurt, cheese, butter, buttermilk, kefir, and koumiss. LAB are also important for human and animal health. Some bile resistant strains survive the passage through the intestinal tract (Rybka & Kailasapathy, 1995) and have beneficial properties when used as probiotics in human and animal nutrition (Havenaar & in't Veld, 1992). LAB refers to a large group of bacteria with many subgroups containing unique traits, but they all have similar metabolic pathways and end-products.

LAB are widespread in nature, and are found primarily in the soil and wherever there are high concentrations of carbohydrates, protein breakdown products, vitamins, and low oxygen. Many LAB are normally found in milk, as well as in meat and vegetables, and certain LAB are normal inhabitants of the human body. Many have been used in food technology to create fermented food products such as yoghurt. LAB are one-celled organisms (prokaryotes). Like other bacteria, LAB produce by binary fission, that is, they form exact replica daughter cells from parent cells (except in rare cases of mutations). They reproduce rapidly, with a doubling-time of 30-90 minutes in optimal conditions (Anon, 1994).

2.2.3.1 Taxonomy of lactic acid bacteria

LAB belong to several genera, which are further divided into species (Table 2.2). Species can be divided into subspecies, variants, and strains, such as in the case of *Lactococcus lactis* ssp. *lactis* var. *diacetylactis* (referred to as *Lc. diacetylactis*). Their ability to ferment specific sugars, their optimal temperature for growth, their nutrient

needs, their sensitivity to salt, and the presence of special enzymes are often distinguishing characteristics of LAB. Methods for classifying between genera, species or strains have evolved from overall morphological appearance and growth conditions to physiological behaviour and to enzymes and metabolic pathways. More accurate techniques involve molecular structure and genetic information. Examples include DNA-DNA and DNA-RNA homology analyses and sequencing of 16S rRNA (Anon, 1994).

As with other fields of science, taxonomy is a continually evolving system. There have been major recent changes, and future revisions are probable. In the taxonomy of LAB, for example, mesophilic bacteria that were originally classified with streptococci have been shifted to the genus Lactococcus. It is important to have universal methods of identification of the LAB used in food fermentation in order to be consistent with regulations and to ensure that strains belong to GRAS species (Anon, 1994).

While LAB are a diverse group, they are classified together because of their common principal end product of metabolism, lactic acid. They can tolerate mild acidic conditions of around pH 4 or several weeks, are gram-positive, and are anaerobic but aerotolerant. Their shapes are primarily cocci (spheres), bacilli (rods), or ovoid. They may also be y-shaped (bifid), as in the case of bifidobacteria (Table 2.2).

Table 2.2 Some characteristics of lactic acid bacteria used in dairy products

Genus	Shape	OptT[a]	Species	Major end-products	Secondary end-products
Streptococcus	Coccus	40-44°C	*S. thermophilus*	L(+) lactic acid	Acetaldehyde, acetone, acetoin, diacetyl [ethanol][b]
Lactobacillus	rod	40-44°C	*L. delbrueckii ssp. bulgaricus*	D(-) lactic acid	Acetaldehyde, acetone, acetoin, diacetyl [ethanol]
			L. helveticus	DL lactic acid	Acetaldehyde, acetic acid, diacetyl [ethanol]
			L. lactis	D(-) lactic acid	Acetaldehyde, acetone, diacetyl [ethanol]
			L. acidophilus	DL lactic	Acetaldehyde [ethanol]
		25-30°C	*L. casei*	L(+) lactic acid	Acetic acid [ethanol]
			L. kefir	DL lactic acid	Acetic acid, acetaldehyde, ethanol, CO_2
Lactococcus	Coccus	25-30°C	*L. lactis*	L(+) lactic acid	Acetaldehyde, acetone, diacetyl, [ethanol]
			L. cremoris	L(+) lactic acid	Acetaldehyde, acetone, diacetyl, [ethanol]
			L. diacetylactis	L(+) lactic acid, acetaldehyde, diacetyl, acetoin, CO_2	Acetone, ethanol
Pediococcus	Coccus	25-30°C	*P. acidilacticici*	DL lactic acid	[acetoin, diacetyl]
Leuconostoc	Ovoid	25-30°C	*Ln. cremoris*	D(-) lactic acid, acetoin, (acetic acid), diacetyl, CO_2	Ethanol
			Ln. dextranicum		
			Ln. lactis		
Bifidobacterium	Rod or bifid	35-38°C	*B. breve*	L(+) lactic acid Acetic acid	Formic acid, succinic acid, acetaldehyde, acetone, acetoin, diacetyl [ethanol]
			B. bifidum		
			B. longum		
			B. infantis		

[a] Opt. T = optimal growth temperature
[b] trace end products are indicated in brackets []
Source: (Anon, 1994).

LAB can be divided into general categories, according either to their metabolic end-products or to their optimal growth temperature. Homofermentative LAB produce lactic acid as their principal end-product (70-90%), whereas heterofermentative bacteria produce other compounds such as acetic acid, CO_2 and ethanol in addition to at least 50% lactic acid (Table 2.2). Mesophilic LAB grow best at a temperature range of 25-30°C, whereas thermophilic bacteria prefer a range of 40-44°C (Table 2.2) and grow at a faster rate than mesophilic bacteria (Anon, 1994).

LAB comprise a wide range of genera including a considerable number of species. It is generally accepted that LAB are Gram-positive and usually catalase-negative bacteria which grow under microaerophilic to strictly anaerobic conditions, and are non-spore forming (Stiles & Holzapfel, 1997). The most important genera of LAB are *Lactobacillus*, *Lactococcus*, *Entercococcus*, *Streptococcus*, *Pediococcus*, *Leuconostoc*, and *Bifidobacterium*. Phylogenetically, Gram-positive bacteria are divided into two major branches. With the exception of the bifidobacteria, all the above-mentioned genera of LAB belong to the Gram-positive phylum with a low G + C (guanine plus cytosine) content (< 50%). Nevertheless, the latter are also considered as LAB because of similar physiological and biochemical properties and the sharing of some common ecological niches such as the gastro-intestinal tract (GIT) (Klein *et al.*, 1998).

Species of these genera can be found in the GIT of man and animal and also in fermented food. Strains used as probiotics usually belong to species of the genera *Lactobacillus*, *Enterococcus*, and *Bifidobacterium*. These LAB are typically chemoorganotrophic and ferment carbohydrates with lactic acid as a major end product. Some physiological characteristics are of interest for their function as probiotics, a pre-condition of which is survival in the gastrointestinal tract. This is based on their resistance to low pH and/or bile and their temperature growth ranges (Fuller, 1989).

2.2.3.2 *Optimal nutrition conditions and metabolism of LAB*

LAB are fastidious microorganisms with complex and varying nutrient needs. They require carbohydrates, principally lactose and glucose, as a carbon and energy source to produce ATP, the energy source of the cell. Milk proteins, peptides, specific amino acids, and nucleic acid derivatives are also necessary to furnish the nitrogen building-blocks for protein synthesis. Several vitamins, in particular the B-vitamins, are often necessary as catalysts for enzyme reactions, but specific vitamin and mineral needs vary between species (Anon, 1994).

Figure 2.1 shows the principal pathways of carbohydrate metabolism in LAB. The major pathway begins with the active transport of lactose through the cell membrane. The lactose or lactose-phosphate is then broken down into glucose and galactose or galactose-phosphate. For homofermentative LAB, such as *S. thermophilus*, most lactobacilli and lactococci, the glucose is further broken down by a series of reactions to pyruvate, which is then transformed into lactate by the enzyme lactate dehydrogenase. LAB cannot synthesize several essential amino acids, and therefore proteolysis of proteins and peptides from the substrate is necessary for their growth. LAB possess proteolytic enzymes that are found in the cell wall, the cell membrane, and the cytoplasm, but only about 2% of casein is broken down during milk fermentation (Figure 2.1) (Anon, 1994).

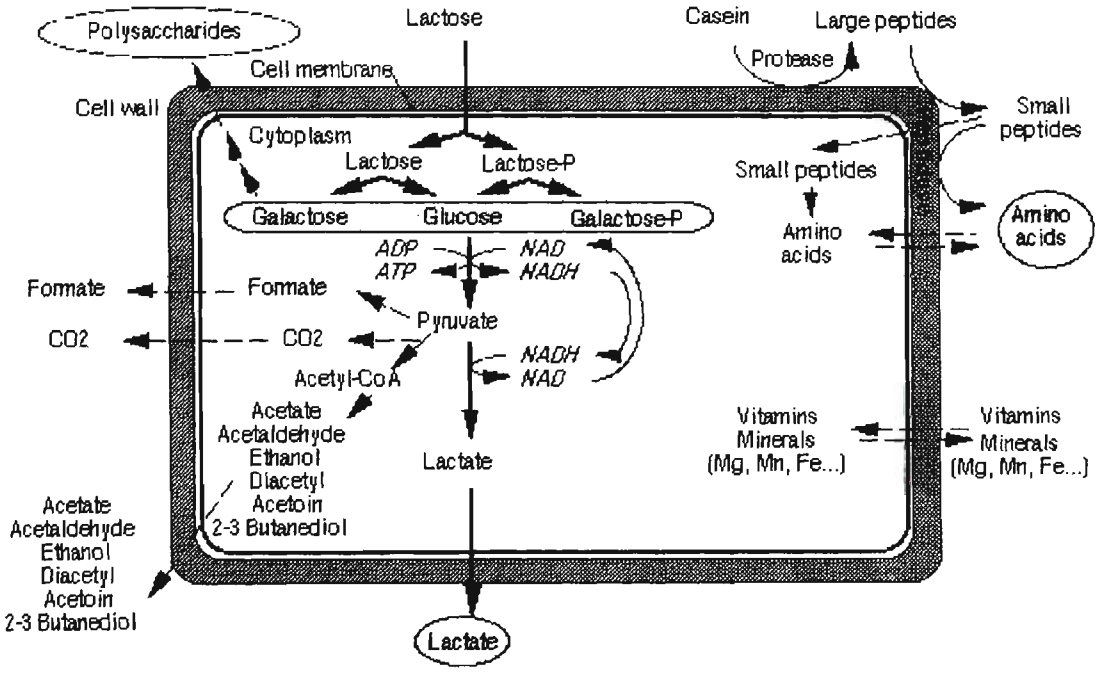

Figure 2.1 Major (->) and minor (-->) metabolic pathways in LAB (ATP/ADP: adenosine tri/di-phosphate, NAD/NADH: oxidized/reduced nicotinamide adenine dinucleotide) (Anon, 1994).

2.2.3.3 Major roles of LAB in fermentation of milk

Fermentation of milk with LAB leads to specific organoleptic characteristics (taste, aroma) of the final product. The metabolism of LAB and the interactions between the selected strains are responsible for the production of various compounds, all of which create these characteristics. Variables such as temperature, pH, the presence of oxygen, and the composition of the milk further contribute to the particular features of a specific product (Anon, 1994).

Fermented milks exhibit a wide variety of textures. They range from liquid drinks such as kefir, koumiss, and acidophilus milk to semi-solid or firm products including yoghurt, filmjolk, villi, dahi, and leben. The characteristic texture of fermented milks is principally related to the production of lactic acid by LAB. As lactic acid accumulates in the milk, it causes a decrease in the pH which leads to the destabilization of the casein micelle through the solubilization of the micellar calcium-phosphate. At a pH of around 4.6-4.7, the casein structure irreversibly unfolds and precipitates, thus causing the

coagulation of milk proteins and the formation of a gel. In addition, certain strains of *S. thermophilus*, *L. delbrueckii* ssp. *bulgaricus*, and other LAB such as *L. cremoris*, and some species of Leuconostoc produce exocellular polysaccharides which modify the texture e.g., by increasing the viscosity, or creating a "ropy" texture (Anon, 1994).

Lactic acid gives fermented milks their slightly tart taste. The other characteristics flavors and aromas are additional results of LAB metabolism. Acetaldehyde provides the characteristic aroma of yoghurt, while diacetyl, produced by *L. diacetylactis* and *L. cremoris* impart a buttery taste to some fermented milks. Acetoin, acetone, lactones, and volatile acids are other important flavors that may be present in certain fermented milks as by-products of metabolism. Additional microorganisms such as yeasts and moulds can also be included in the culture to provide unique tastes. Alcohol and CO_2 produced by yeasts contribute to the refreshing, frothy taste of kefir, koumiss, and leben. Other manufacturing techniques such as removing the whey or adding flavors, also contribute to the large variety of available products (Anon, 1994).

According to the definition of Codex Alimentarius, yoghurt is the result of milk that has been fermented by *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus*, both of which are thermophilic and homofermentative. In certain countries, according to specific regulations, other LAB such as *L. acidophilus* and bifidobacteria can be added to the culture. Strain selection and processing conditions are responsible for different characteristics of the final product. LAB are grown in milk under defined conditions to produce a final product containing 100-1000 million live bacteria (referred to as colony forming units, (cfu)) per mL. This level may decrease by a factor of 10-100 during storage, depending on temperature and time. A typical yoghurt has a pH of around 3.9-4.3, and 20-30% of the lactose has been converted to lactic acid (Anon, 1994).

There is a symbiotic relationship between *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* where each species of bacteria stimulates the growth of the other. *L. delbrueckii* ssp. *bulgaricus* stimulates the growth of *S. thermophilus* by liberating amino acids and peptides from milk proteins, which enable *S. thermophilus* to grow faster in the

early part of incubation. *S. thermophilus* in turn produces formic acid which stimulates the growth of *L. delbrueckii* ssp. *bulgaricus*. This interaction, known as "protocooperation" results in a shortened fermentation time and a product with different characteristics than a product fermented with a single species (Anon, 1994).

2.2.3.4 Selecting lactic acid bacteria starter cultures

Starter cultures refer to the specific LAB which are used to inoculate milk and whose metabolism leads to the characteristics of the fermented milk product. There are two types of starter cultures. The first traditional method contains complex, poorly defined mixtures of bacterial strains. The second type is called a "defined" or "selected" culture, and contains one or more identified pure strains with specific known properties. Specific criteria for culture selection include the rate of producing acid, their capacity for polysaccharide production, their limited proteolysis (which sometimes leads to bitter compounds) and their production of aromatic (flavor) compounds (Anon, 1994).

Another criterion for starter selection is based on the ability of LAB to increase the nutritional and/or physiological value of the food, thus resulting in health properties. For example, in certain countries, *L. acidophilus*, bifidobacteria, or *L. casei* are included in the starter because of their possible beneficial effects in the gastrointestinal tract. Other goals may include improving the vitamin profile (Anon, 1994).

2.2.4 Probiotic bacteria

Members of the genera *Lactobacillus* and *Bifidobacterium* which have a long and safe history in the manufacture of dairy products and are also found among gastrointestinal microflora have been traditionally included in probiotic products. The recent well-documented clinical studies clearly support health claims of certain strains including *Lactobacillus johnsonii* La1, *Lactobacillus rhamnosus* GG, *Lactobacillus casei* Shiroto strain, *Lactobacillus acidophilus* NCFB 1487 and *Bifidobacterium lactis* Bb12 (Vaughan & Mollet, 1999).

2.2.4.1 Genus Bifidobacterium

Bifidobacteria are normal inhabitants of the human gastrointestinal tract. Recent *in vivo* scientific studies, using animals or human volunteers, have shown that consumption of live bifidobacteria have an effect on the gut microflora. Selected strains survive stomach and intestinal transit and reach the colon in abundant numbers. The gut microflora is subsequently affected, as measured directly by fecal enumerations and enzyme assays or indirectly by assessing gut transit time. The mechanism of action remains to be elucidated. Bifidobacteria are normal inhabitants of the human and animal gut, and newborns are colonized within days after birth. The population seems to be relatively stable until advanced age (Figure 2.2) when it has been reported to decline. Although the population of bifidobacteria in the intestines is relatively stable, it is influenced by diet, antibiotics, stress etc (Anon, 1994).

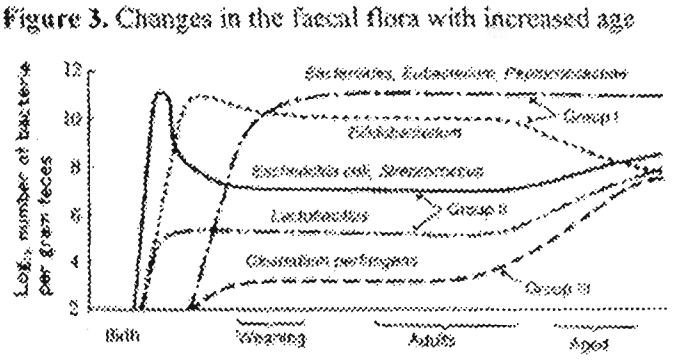

Figure 2.2 Differences in species of bacteria in human feces of different ages (Anon, 1994).

2.2.4.1.1 Taxonomy of Bifidobacterium ssp.

Bifidobacteria were first isolated and described in 1899-1900 by Tissier, who described them as rod-shaped (Figure 2.3), non-gas-producing, anaerobic microorganisms with bifid morphology, present in the faeces of breast-fed infants, which he termed *Bacillus bifidus*. Bifidobacteria are generally characterized as gram-positive, non-spore forming, non-motile and catalase-negative anaerobes (Sgorbati *et al.*, 1995; Vijayendra & Gupta, 1992; Gomes & Malcata, 1999). They have various shapes including short, curved rods,

club-shaped rods and bifurcated Y-shaped rods. Bifidobacteria are anaerobes with a special metabolic pathway which allows these LAB to produce acetic acid as well. They frequently have special nutritional requirements, thus often making these bacteria difficult to isolate and grow in the laboratory (Anon, 1994).

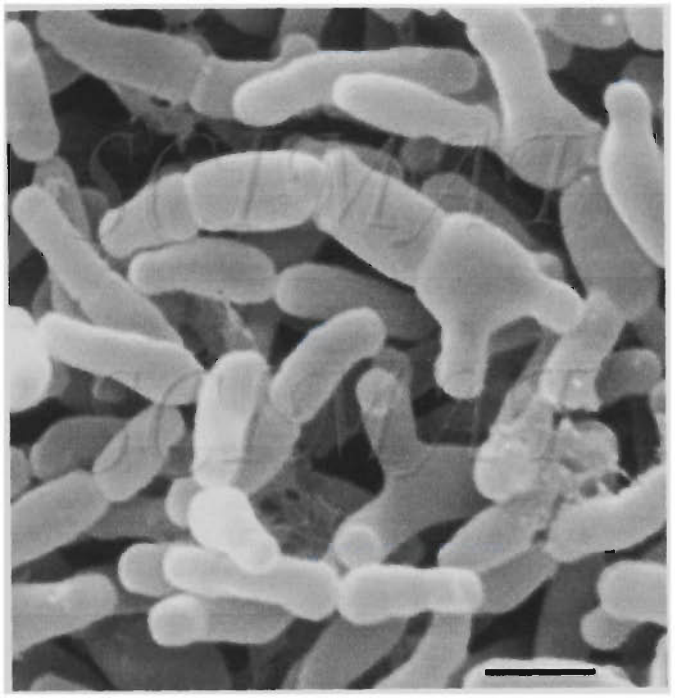

Figure 2.3 ***Bifidobacterium breve*, (Bar 1μm, Anon, 2000).**

The taxonomy of bifidobacteria has changed continuously since they were first isolated. They have been assigned to the genera *Bacillus, Bacteroides, Nocardia, Lactobacillus* and *Corynebacterium* among others, before being recognized as a separate genera in 1974. Presently, 29 species are included in the genus *Bifidobacterium*, 10 of which are from human sources (i.e., dental caries, faeces and vagina), 17 from animal intestinal tracts or rumen, two from wastewater and one from fermented milk (Table 2.3).

Table 2.3 List of species of the genera *Bifidobacterium*[a] *ssp.*

Bifidobacterium species	
B. adolescentis[q]	*B. infantis*[a]
B. angulatum[a]	*B. lactis*
B. asteroids	*B. longum*[a]
B. bifidum[a]	*B. magnum*
B. boum	*B. merycicum*
B. breve[a]	*B. minimum*
B. catenulatum[a]	*B. pseudocatenulatum*[a]
B. choerinum	*B. pseudolongum*
B. coryneforme	*B. pullorum*
B. cuniculi	*B. ruminantium*
B. dentium[a]	*B. saeculare*
B. gallicum	*B. subtile*
B. gallinarum	*B. suis*
B. globosum[a]	*B. thermophilum*
B. indicum	

[a] Species isolated from human species
Source Gomes & Malcata, 1999.

Bifidobacteria are phylogenetically grouped in the actinomycetes branch of gram-positive bacteria (Gomes & Malcata, 1999), that is characterized by a high guanine plus cytosine (G + C) content, which varies from 54 to 67 mol% (Table 2.4). In addition, there are notable differences in physiological and biochemical properties, including cell-wall constituents (Table 2.4). They are saccharolytic organisms that produce acetic and lactic acids without generation of CO_2, except during degradation of gluconate. Besides glucose, all bifidobacteria from human origin are also able to utilize galactose, lactose and, usually, fructose as carbon sources. *Bifidobacterium* ssp. are, in some instances, also able to ferment complex carbohydrates; a recent study (Crociani *et al.*, 1994), in which 290 strains of 29 species of bifidobacteria from human and animal origin were surveyed for their ability to ferment complex carbohydrates, has confirmed this potential. The substrates fermented by the largest number of species were D-galactosamine, D-glucosamine, amylose and amylopectin. Porcine gastric mucin was fermented only by *B. bifidum*, whereas *B. infantis* was the only species that could ferment D-glucuronic acid.

The optimum pH for the growth of bifidobacteria is 6-7, with virtually no growth at pH 4.5-5.0 and below or at pH 8.0-8.5 and above. Optimum growth temperature is 37-41°C, with maximum growth at 43-45°C and virtually no growth at 25-28°C or below. Comprehensive details of their biology are available in the extensive reviews by Rasic and Kurmann (1983) and Sgorbati *et al.* (1995).

Table 2.4 Physiological and biochemical characteristics of *Bifidobacterium* ssp. and *Lactobacillus acidophilus*[a]

Character	*Bifidobacterium* ssp.	*Lactobacillus acidophilus*
Physiology	Anaerobic	Microaerophilic
Cell wall composition		
Peptidoglycan type	Variable, basic amino acid in the tetrapeptide is either ornithine or lysine, various types of cross-linkage	Lys-D Asp
Phospholipid composition/Techoic acid	Polyglycerophospholipid and its lyso derivatives, alanylphosphatidylglycerol, lyso derivatives of diphosphatidylglycerol	Glycerol
DNA-base composition Mol% G + C (guanine + cytosine)	55-67	34-37
Lactic acid configuration	L	DL
Sugar metabolism	Heterofermentative	Homofermentative

[a] Adapted from Kurmann and Rasic (1991) and Mital and Garg (1992)

2.2.4.1.2 Bifidogenic factors and growth factors

There are clear differences between bifidogenic factors and growth factors for bifidobacteria in terms of nature and function. Bifidogenic factors are defined as 'compounds, usually of a carbohydrate nature, that survive direct metabolism by the host and reach the large bowel or cecum, where they are preferentially metabolized by bifidobacteria as source of energy'. Bifidogenic factors may fall under the new concept of prebiotics, which are defined as non-digestable 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, and which may thus improve the health of the host. In contrast, growth factors are compounds that promote the growth of bifidobacteria *in vitro* but cannot be delivered to the large bowel or cecum or selectively promote proliferation of

bifidobacteria (Modler, 1994). Studies on the sources and applications for either of these types of compounds have been well documented (Modler, 1994), yet comprehensive reviews of the state of the art in this field are still lacking. Table 2.5 and Table 2.6 summarize established knowledge and more recent developments in the isolation, identification and application of bifidogenic and growth factors, respectively.

Table 2.5 Origin and effects of possible bifidogenic factors for *Bifidobacterium* ssp.[a]

Name	Origin	Strains studied	Effects	Reference
Casein bifidus factor (CBF)	Bovine casein submitted to acid and enzyme (papain and pepsin) hydrolysis	*Bifidobacterium bifidum*	Growth promotion, best with acid CBF; CBF supplies both peptides and carbohydrates necessary for growth	Zhikowski & Ziajka (1986)
Human κ-casein and glycomacropeptide derived therefrom	Intact κ-casein from human milk and hydrolysed with chymosin and pepsin	*Bifidobacterium infantis* S12	Small growth-promoting activity of intact κ-casein enhanced upon enzymatic hydrolysis	Azuma *et al.* (1984)
K-Casein enzymatic digest	Bovine milk casein digested by trypsin	*Bifidobacterium* ssp. (*B. bifidum*, *B. longum*)	Growth promoting activity in fully synthetic medium associated with disulfide/sulfhydryl (cystine) residues and a certain tryptic peptide-unidentified bifidogenic factor	Poch & Bezkorovainy (1991)
Casein macropeptide (CMP)	Bovine milk	*Bifidobacterium* ssp.	Growth promoting activity	Abd-El-Salam *et al.* (1996)
N-acetylneuronic acid (Neu-Ac)-containing substances (NeuAc, sialyl lactose, glycomacropeptide) transgalactosylated oligosaccharides galactosyl galactose, galactosyl glucose fructans	Bovine milk	*Bifidobacterium* ssp. and lactobacilli	Growth promoting activity of *B. breve*, *B. bifidum*, *B. infantis*	Idota *et al.* (1994)
	Ash-free white powder from tubers of Jerusalem artichokes	*Bifidobacterium* ssp. (*B. infantis* ATCC 15697, *B. adolescentis* ATCC 15703, *B. longum* ATCC 15707)	Growth promoting activity	Yamazaki & Matsumoto (1994)
Xylooligosaccharides (xylobiose)	Wheat bran, corn cobs, aspen, wheat straw	*Bifidobacterium* ssp.	Increase of intestinal bifidobacteria with 1-2 g/d	Okazaki *et al.* (1990)
Oligosaccharides	Onion, garlic, chicory root, burdock, asparagus, Jerusalem artichoke, soybeans, wheat bran	*Bifidobacterium* ssp.	Proliferation of bifidobacteria and suppression of putrefactive bacteria	Yamada *et al.* (1993) Tomomatsu (1994)

[a] Adapted from Gomes *et al.* (1999)

Table 2.6 Origin and effects of possible growth factors for *Bifidobacterium* ssp. [a]

Name	Origin	Strains studies	Effects	Reference
Depolymerized alginates	Sodium alginate (sea-weed Lessonia ssp.) depolymerized by bacterial alginate lyase	*Bifidobacterium* ssp. (*B. breve*, *B. longum*, *B. adolescentis*, *B. bifidum*, *B. infantis*)	Growth promoting activity in skim-milk	Akiyama *et al.* (1992)
Casein hydrolyzates	Alcalase, chymotrypsin and trypsin hydrolysis of casein followed by ultrafiltration (MW cut-off 30 kDa) commercial hydrolyzates (N-Z Case)	*Bifidobacterium* ssp. (*B. bifidum* var. *pennsylvanicus*, *B. adolescentis*, *B. breve*, *B. infantis*, *B. longum*)	Growth stimulation of *B. infantis* and *B. breve* in synthetic medium; commercial hydrolysate promoted better growth of all strains that ultrafiltered hydrolyzates	Proulx *et al.* (1992)
Casein hydrolyzates	Alcalase, chymotrypsin and trypsin hydrolysis of casein followed by two-step ultrafiltration (MW cut-off 1kDa)	*Bifidobacterium* ssp. (*B. breve*, *B. infantis*, *B. longum*)	Trypsin peptide fraction at 2% in synthetic Garches medium promoted best strain growth; alcalase amino acid fraction at 1-2% repressed growth, and acid production by *B. breve* and *B. longum*	Proulx *et al.* (1994)
Bienzyme hydrolyzed solutions	Soybean and sword jackbean (Canavlia gladiata) China	*Bifidobacterium* ssp. for beverage production	Growth stimulation reduction in renneting time	Yang *et al.* (1996)
Milk hydrolyzates	Proteinase (MHP) and neutrase (NHN) hydrolysis of UHT milk to different degrees	*Bifidobacterium lactis* and *Lactobacillus acidophilus*	24-h hydrolysis of MHP at 5% promoted best growth of *B. lactis* in bovine, ovine and caprine milks *L. acidophilus* was not affected by either MHP or MHN; free amino acid fraction alone was less efficient than mixture of amino acids and low molecular weight peptides (<500 Da) of 24-h MHP	Gomes & Malcata (1998)

[a] Adapted from Gomes *et al.* (1999)

Within the range of compounds investigated, derivatives of human and bovine milks (Table 2.6) have proven good candidates for the enhancement of growth of bifidobacteria *in vitro*. According to Poch and Bezkorovainy (1991), growth-promoting activity of bovine milk κ-casein was ascribed to its conjugated cystine residues. This observation has been confirmed and refined in a study aimed at comparing the growth-promoting effects of various biological materials on *B. longum* (Ibrahim & Bezkorovainy, 1994) using a synthetic medium. All tentative growth-promoting factors studied failed to exhibit their growth-promoting activity when their disulfide bonds were reduced or alkylated, thus suggesting that their composition in sulfur-containing peptides might be the key to the observed effect. The major whey proteins, α-lactlabumin and β-lactoglobulin, were also found to be excellent growth promoters, a trait validated after the studies by Petschow and Talbott (1990) on the effectiveness of whey fractions of both human milk and bovine milk. Yeast extract, a commercial product, was found to be an effective growth promoter, and is most often used at concentrations that vary between 0.1 and 0.5% (v/v). A previous study (Roy & Dussault, 1990) has shown, however, that addition of yeast extract at 0.25% (v/v) did not stimulate growth of *B. infantis*, even though acid production was enhanced. It further demonstrated the good growth-promoting activity of β-glycerophosphate in the presence of cysteine for *B. bifidum* and *B. infantis*, but not for *B. longum* ATCC 15708.

Other biological compounds identified as growth factors for bifidobacteria include threonine, cysteine, enzyme-treated chlorella, peptone and trypticase rots (coenzyme A), potatoes and corn, and commercial casein hydrolysates that are, in general, produced by breaking down casein with proteases, peptidases or strong acids to different yields. Proulx *et al.* (1992, 1994) claimed that peptides obtained from casein hydrolysates might be a preferable source of nitrogen than free amino acids for dairy-related bifidobacteria (Table 2.6). Apart from that, the development of hydrolysates with better quality at lower cost is essential. Gomes *et al.* (1998) expanded on this work using milk (as economical source), hydrolysed to several extents by two alternative proteinases, as nitrogen source to promote growth of, and acidification by *B. lactis* and *L. acidophilus*. These authors established that both the nature of the enzyme and the degree of hydrolysis are important

criteria towards attainment of consistent growth of the aforementioned strains. This observation confirms the growth-limiting characteristics of available nitrogen in the form of low molecular weight peptides (<500Da) and free amino acids that are more easily assimilated by *B. lactis* (Table 2.6).

2.2.4.1.3 Growth performance and effect of substrate on growth

The fact that bifidobacteria can grow in a semi-synthetic medium containing only lactose, three free amino acids (cysteine, glycine and tryptophan), several vitamins and nucleotides, and some minerals contrasts with the generally recognized fastidiousness of lactobacilli with regard to nutritional requirements. Certain strains were found to grow on a simplified medium comprised of lactose (as fermentable carbohydrates), buffers, minerals, ammonium salts, cysteine, and the vitamins biotin and calcium pantothenate (Gomes and Malcata, 1999). *Bifidobacterium bifidum* var. *pennsylvanicus*, a mutant strain, requires pantothenate and N-acetylglucosamine-containing saccharides for growth and synthesis of its cell wall. The *B. bifidum* strain discovered by Tissier in 1899, as well as some other bifidobacteria, require a nitrogen source (i.e. peptides), yet neither pantothenate nor N-acetylglucosamine-containing saccharides are essential. A striking difference between lactobacilli and some strains of bifidobacteria is the ability of the latter to grow in a medium containing nitrogen in ammonium form: the remaining bifidobacteria strains require nitrogen from organic sources (Kurmann, 1998). Evidence indicates that the nutritional needs of bifidobacteria are species-dependent, so any new strain discovered or produced needs to be extensively studied in terms of its minimal nutritional requirements; this is the case of the newly identified *Bifidobacterium lactis* strain (Gomes *et al.*, 1998).

2.2.4.2 Genus Lactobacillus acidophilus

In 1990, Moro was the first researcher to isolate facultative straight rods from the faeces of breast-fed infants, which he typified as *Bacillus acidophilus*, a generic name for intestinal lactobacilli. Lactobacilli are in general characterized as gram-positive, non-sporeforming and non-flagellated rods or coccobacilli (Figure 2.4) (Hammes & Vogel, 1995). *L. acidophilus* are obligately homofermentative facultatively anaerobic rods

whose major end-product is lactic acid. They occur naturally in the gastrointestinal tract of humans and animals, in the human mouth and vagina, and in some traditional fermented milks, such as kefir (Anon, 1994).

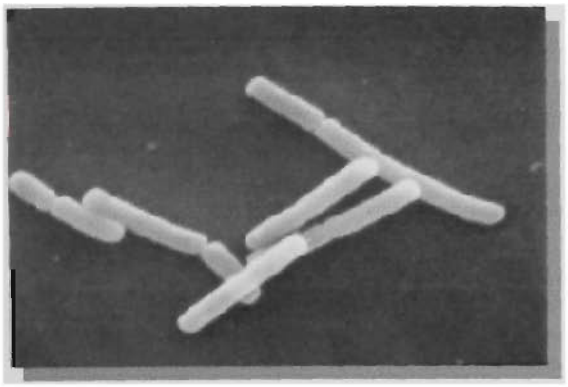

Figure 2.4 ***Lactobacillus acidophilus*** **(Anon, 2003).**

The G + C content of their DNA is usually between 32 and 51 mol%. They are either aerotolerant or anaerobic and strictly fermentative. Glucose is fermented predominantly to lactic acid in the homofermentative case, or equimolar amounts of lactic acid, CO_2 and ethanol (and/or acetic acid) in the heterofermentative counterpart (Mital & Garg, 1992). Because *L. acidophilus* is a genetically heterogeneous species, their classification has been difficult. Other species have in fact sometimes been incorrectly labeled as *L. acidophilus* (e.g., some strains of *Lactobacillus casei*). DNA-DNA homology led to the identification of six major species (*L. acidophilus*, *L. crispatus*, *L. amylovorus*, *L. gallinarum*, *L. gasseri*, and *L. johnsonii*) which exhibit clear observable differences but constitute the same *L. acidophilus* group. A new more rapid technique called random amplified polymorphic DNA (RAPD)-PCR has recently been developed that can differentiate between the various strains of each of the *L. acidophilus* species (Anon, 1994).

At present, 56 species of the genus *Lactobacillus* have been recognized (Table 2.7). Of these microorganisms, the most commonly suggested for dietary use are *Lactobacillus acidophilus* strains, the definition of which has changed recently.

Table 2.7 List of species (by alphabetical order) of the genera *Lactobacillus*[a]

Lactobacillus species			
L. acetotolerans	*L. curvatus*	*L. intestinalis*	*L. plantarum*[a]
L. acidophilus[a]	*L. delbrueckii*	*L. jensenii*[a]	*L. reuteri*[a]
L. alimentarius	*L. farciminis*	*L. johnsonii*	*L. rhamnosus*[a]
L. amylophilus	*L. fermentum*[a]	*L. kefir*	*L. ruminus*
L. amylovorus	*L. fructivorans*	*L. kefiranofaciens*	*L. sake*
L. avarius	*L. fructosus*	*L. malefermentans*	*L. salivarus*[a]
L. bifermentans	*L. gallinarum*	*L. mali*	*L. sanfrancisco*
L. brevis[a]	*L. gasseri*[a]	*L. minor*	*L. sharpeae*
L. buchneri[a]	*L. graminis*	*L. murinus*	*L. suebicus*
L. casei ssp. *casei*[a]	*L. halotolerans*	*L. oris*[a]	*L. vaccinostercus*
L. collinoides	*L. hamsteri*	*L. parabuchneri*[a]	*L. vaginalis*[a]
L. confuses	*L. helveticus*	*L. paracasei*[a]	*L. viridescens*
L. coryniformis	*L. hilgardii*	*L. pentosus*	
L. crispatus[a]	*L. homohiochii*	*L. pontis*	

[a] Species isolated from human sources
Source: Gomes and Malcata (1999).

Comprehensive genetic studies have shown that the original species actually consists of six DNA homology groups, including *L. crispatus*, *L. gallinarum*, *L. gasseri*, *L. amylovorus* and *L. johnsonii*. Although these species are well defined, difficulties are often encountered in allocating newly isolated strains of these groups. Investigations based on agglutination tests, cell wall antigen and electrophoretic and antigenic characteristics of D- and L-lactate dehydrogenase also point toward heterogeneity of these species (Mital & Garg, 1992). *Lactobacillus 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 0.6-0.9μm in width and 1.5-6.0μm in length. It is non-flagellated, non-motile and non-sporeforming, and is intolerant to salt. This microorganism does not contain cytochromes and, therefore, is benzidine negative. In addition, it is microaerophilic, so surface growth on solid media is generally enhanced by anaerobiosis or reduced oxygen pressure and 5-10% CO_2. The physiological and biochemical characteristics of this microorganism are given in Table 2.4. Most strains of *L. acidophilus* can ferment amygdalin, cellobiose, fructose, galactose, glucose, lactose, maltose, mannose, salicin, sucrose, trehalose and aesculine (Gomes *et al.*, 1999). Lactose is virtually the only sugar present in milk, yet *L. acidophilus* has been reported to utilize

sucrose more effectively than lactose; such observations may be ascribed to differences in β-galactosidase (EC 3.2.1.23) and β-fructofuranosidase (EC 3.2.1.26) activities. While β-fructofuranosidase is a constitutive enzyme, β-galactosidase may be induced in *L. acidophilus* (Nielsen & Gilliland, 1992). Moreover, both glucose and fructose moieties of sucrose are utilized by *L. acidophilus*, whereas the galactose moiety of lactose cannot be metabolized to an appreciable degree. The glucose moiety is metabolized via the Embden-Meyerhof-Parnas pathway with lactic acid as essentially the sole end product. The yield of lactic acid is 1.8mol/mol glucose, accompanied by minor amounts of other compounds. Acetaldehyde, a carbonyl flavouring molecule, may also result from metabolism of lactose, although in some instances it may be produced from metabolism of nitrogen-containing substances, e.g. threonine; a very high activity of threonine aldolase has been found in *L. acidophilus*. Growth of *L. acidophilus* may occur at as high as 45°C, but optimum growth occurs within 35-40°C. Its acid tolerance varies from 0.3% to 1.9% titratable acidity, with an optimum pH lying at 5.5-6.0 (Gomes *et al.*, 1999).

2.2.4.2.1 Growth performance and effect of substrate on growth

Lactobacilli have complex growth requirements. They require low oxygen tension (Klaver *et al.* 1993), fermentable carbohydrates, protein and its breakdown products, a number of vitamins of the B-complex, nucleic acid derivatives, unsaturated free fatty acids, and minerals such as magnesium, manganese and iron for their growth. Increased amount of thiol groups present in whey protein-enriched milks favours the growth of *L. acidophilus*, whereas peptone and trypsin stimulate its acid production (Kurmann, 1998). Addition of tomato juice (as a source of simple sugars, minerals and vitamins of the B-complex) to skimmed milk has provided evidence for enhancement of both growth of (i.e. higher viable counts and shorter generation times) and activity by (i.e. improved sugar utilization and lower pH) *L. acidophilus* (Babu *et al.*, 1992). These essential nutrients should, therefore, be available in the medium for establishment of a predominant microflora of lactobacilli.

2.2.4.3 *Milk as an undefined nutrient medium for probiotic bacteria*

Bifidobacteria are used in milk fermentation to a limited extent because of their slow growth in that matrix which, in many instances, may be considered as an artificial medium. However, milk is an essentially satisfactory medium because it contains all essential nutrients, except that amino acids and small peptides are present at insufficient concentrations (ca. 0.1g/L) to support extended growth of bifidobacteria (Rasic & Kurmann, 1983; Gomes *et al.*, 1998). Nevertheless, cases of adaptation to milk upon multiple transfers has been reported (Kurmann, 1998). Comparison of various milk types (bovine, ovine and caprine milks) with respect to their available nitrogen fraction has also been exploited in an attempt to further probe the influence of the raw material upon selected probiotic strains, but the higher protein and vitamin contents of ovine milk were not sufficient to sustain growth of *B. lactis* at the high rates required. Moreover, the excess of fatty acid residues present in caprine milk were indicated as responsible for the poor growth of *L. acidophilus* (Gomes *et al.*, 1999).

In general, bifidobacteria grow better in rich synthetic media, viz. TPY and MRS broths, than in milk; however, those media are complex and costly for large-scale propagation of bifidobacteria. Moreover, unless the cells harvested from such media are extensively washed before incorporation, they may confer off-flavours to the finished dairy products. To manufacture a quality product, both in terms of texture and viability of bifidobacteria, a milk-based medium is usually required because the casein content of milk protein is higher that that of synthetic media (which are generally low in solids). Thus, improvement of growth conditions for the different strains of bifidobacteria in milk, in particular via addition of various easily-available nitrogen sources or redox potential-studies (Gomes *et al.*, 1998; Klaver *et al.*, 1993; Ventling & Mistry, 1993), and using less fastidious, mildly-acidifying strains can thus be claimed as key candidates for successful bifidobacteria-containing (bio) products.

L. acidophilus tends to grow slowly in milk, leading to the risk of overgrowth of undesirable microorganisms, and strains that do not grow well tend to produce unpleasant flavors. Ironically, most strains of *L. acidophilus* do not survive well in fermented milk

due to the low pH (they are not acid-loving, after all), and it is difficult to maintain large numbers in the product. For these reasons, fermented milk products containing *L. acidophilus* are often produced in conjunction with other cultures. The various cultures are either fermented together or fermented separately and then mixed. Examples include adding *L. acidophilus* to yoghurt cultures (*S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus*), to *Lactococcus lactis*, or to bifidobacteria and *Pediococcus acidilactici* (Mital & Garg, 1992).

L. acidophilus and *Bifidobacterium* ssp. grow poorly in milk even though they show a high level of β-galactosidase activity. The reason of such behaviour might be related to the low concentration of free amino acids and small peptides in milk, which would be insufficient to support the bacterial growth. Therefore, it is assumed that the ability of *L. acidophilus* and *Bifidobacterium* ssp. to grow in milk would depend on the presence of an efficient proteolytic system to hydrolyze milk proteins or the addition of proteolytic organisms that would be able to support their growth.

2.3 Yoghurt bacteria

2.3.1 Genus Streptococcus thermophilus

Streptococcus thermophilus (Figure 2.5) was originally described by Orla-Jensen (1919) and stands apart from the other streptococci and especially lactic streptococci, at present designated as lactococci. It is exclusively isolated from the dairy environment, ferments only few carbohydrates, i.e. lactose, sucrose, glucose and sometimes galactose, and is characterized by its thermoresistance and a rather high growth temperature which may reach 50-52°C.

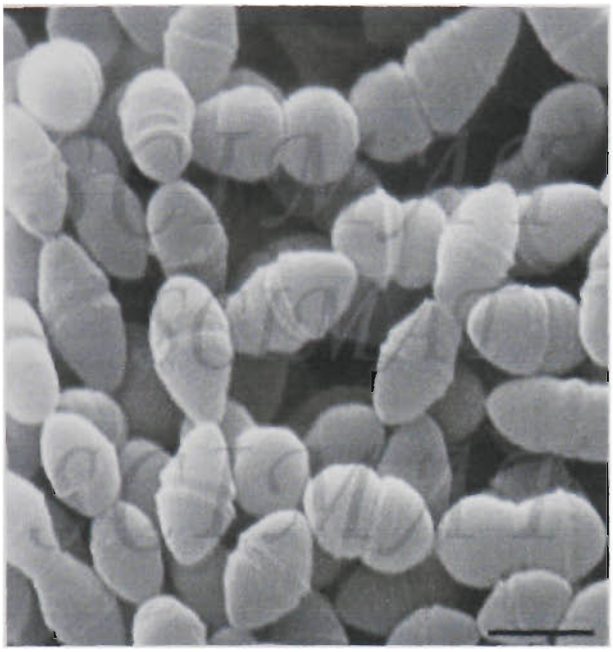

Figure 2.5 ***Streptococcus thermophilus*** **is one of the bacterial strains used to make yoghurt (Bar, 1µm, Anon, 2000).**

DNA-DNA homology studies might supply more information on this bacterium and may question its classification. The guanosine plus cytosine content (mol G + C %) of DNA ranges from 37.2-40.3% according to Farrow and Collins (1984). These authors obtained 61-78% homology between DNA of several *S. thermophilus* strains and a DNA probe of *Streptococcus salivarius* (the homology between DNA of *S. salivarius* strains and a DNA probe of *S. thermophilus* ranged from 67-91%). They thus confirmed the high DNA homology (70-100%) observed in an earlier study by Kilpper-Bätz *et al.* (1982) for 2 strains of each species. On the basis of these results, Farrow and Collins (1984) proposed that *S. thermophilus* should be reclassified as a subspecies of *S. salivarius* despite the large phenotypic differences between those 2 bacteria (Zourari & Desmazeaud, 1992).

2.3.2 Genus Lactobacillus delbrueckii ssp. *bulgaricus*

This lactobacillus was first described by Orla-Jensen (1919) and named *Thermobacterium bulgaricum*. It is presently considered as one of the subspecies of *Lactobacillus delbrueckii*. Two other subspecies, subsp *delbrueckii* and subsp *lactis*, also belong to this group. The old terminology *Lactobacillus leichmannii* is no longer in use. *L. delbrueckii* ssp. *bulgaricus* (Figure 2.6) is homofermentative, ferments few

carbohydrates, i.e. glucose, lactose, fructose, and sometimes galactose or mannose, and has a high growth temperature (up to 48 or 50°C). Its DNA mol G + C % ranges from 49 to 51%. Simonds *et al.* (1971) obtained a DNA homology of 86% between the former *L. bulgaricus* and *L. lactis* species, but only 4.8% with *L. helveticus* var *jugurti.* This was confirmed by Deilaglio *et al.* (1973) who obtained <7% DNA homology between *L. bulgaricus* and *L. helveticus* or *L. helveticus* var *jugurti.*

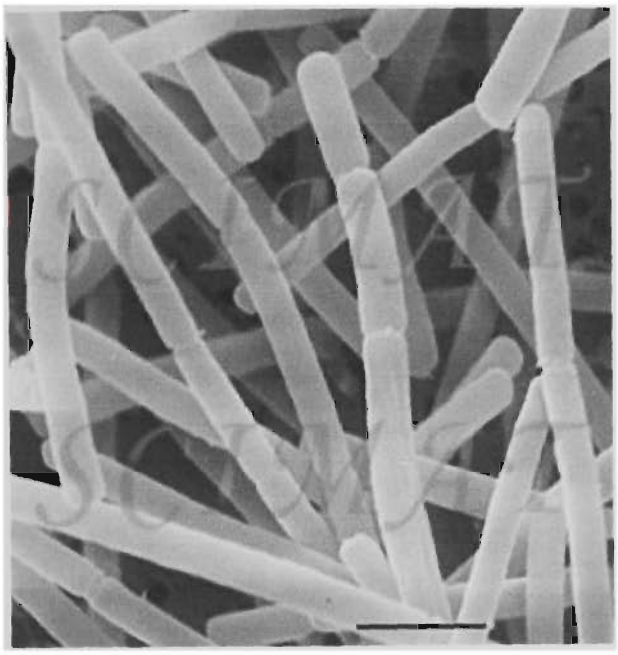

Figure 2.6 ***Lactobacillus delbrueckii* ssp. *bulgaricus* (Bar, 2µm, Anon, 2000).**

The study of Weiss *et al.* (1983) showed 80 to 100% homology (90-100% for type-strains) between the former *L. bulgaricus*, *L. leichmannii*, *L. lactis* and *L. delbrueckii* species. DNA homology led to the reclassification of these 4 lactobacilli as subspecies of *L. delbrueckii* in the most recent classification of Bergey's Manual (Zourari & Desmazeaud, 1992). A specific DNA probe for *L. delbrueckii* has been developed recently, which makes it possible to differentiate this species from other lactobacilli, lactococci and propionibacteria.

Lactobacillus delbrueckii ssp. *bulgaricus* is a lactic acid bacterium widely used for the production of dairy foods such as yoghurts and fermented milks. During its growth, this homofermentative bacterium produces lactic acid, which leads to acidification of the medium to approximately pH 3.8. *Lactobacillus delbrueckii* ssp. *bulgaricus* is a

neutrophilic microorganism which must adapt to the increasing acidity in order to survive. Thus, acidity is one of the major stresses encountered by the bacterium in yoghurts (pH 4.3-4.5) as well as in the stomach (pH 1.5-2.0). However, little is known about the physiology of *Lactobacillus delbrueckii* ssp. *bulgaricus*, notably concerning its response to fermentation-related and gastrointestinal stresses (Lim *et al.*, 2000).

2.3.3 Important metabolic activities of S. thermophilus and L. delbrueckii ssp. bulgaricus for yoghurt manufacture

The role of streptococci and lactobacilli in yoghurt manufacture can be summarized as follows: milk acidification, synthesis of aromatic compounds, development of texture and viscosity. The latter aspect is required mainly for stirred and liquid yoghurts. Thus for industrial yoghurt manufacture, starter selection takes into account these three properties. Evaluation of acidification properties is difficult because of the high buffering capacity of milk and the lack of a standard procedure. Efforts have been made to develop new methods for an objective evaluation of strains in replacement of former procedures (e.g. that of Accolas *et al.*, 1977) based on the measurement of titratable acidity in milk. The evaluation of aroma formation is generally based on the production of acetaldehyde, a major aromatic compound of yoghurt, whereas the thickening character is based on measurements of milk viscosity (Zourari *et al.*, 1992; Zourari *et al.*, 1991).

2.3.3.1 Acid production and carbohydrate metabolism

The main role of *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* in yoghurt manufacture is to acidify milk by producing a large amount of lactic acid from lactose. Lactic acid reduces the pH of the milk and leads to a progressive solubilization of micellar calcium phosphate. This causes the demineralization of casein micelles and their destabilization, which generates the complete precipitation of casein in a pH range of 4.6-4.7 (Fox, 1989). In addition, lactic acid provides yoghurt with its sharp, acid taste and contributes to flavour.

Lactic acid production may also occur during yoghurt storage at low temperature. This is termed as post acidification. It may lead to an excessive acidification which affects the

organoleptic properties of the product. This activity depends on the strains used and especially on the lactobacilli (Zourari & Desmazeaud, 1992).

2.3.3.2 *Proteolytic activity and amino acids in milk and yoghurt*

In yoghurt, proteolysis is not a determinant for organoleptic properties, but it is an important factor for the selection of strains for cheese making. On the other hand, proteolytic activity is greatly involved in both nutrition and interactions of yoghurt bacteria, since lactic acid bacteria cannot synthesize essential amino acids. Therefore, they require an exogenous nitrogen source and utilize peptides and proteins in their growth medium by more or less complete enzyme systems.

S. thermophilus primarily requires glutamic acid, histidine and methionine, as well as cystine, valine, leucine, isoleucine, tryptophan, arginine and tyrosine for growth (Shankar & Davies, 1977). The uptake of branched-chain amino acids has been studied. It is an active transport which requires an exogenous energy source, depends on temperature and pH and is inhibited by L-cysteine.

The free amino acid content of cow's milk generally does not exceed 10 mg/100 mL (Rasic & Kurmann, 1978; Alm, 1982c). In yoghurt, the free amino acid pattern depends on the type of milk (animal species, season), its heat treatment, manufacturing techniques, bacterial strains used and storage conditions (Amer & Lammerding, 1983). The free amino acid content generally increases in yoghurt compared to that of milk. *L. delbrueckii* ssp. *bulgaricus* appears to be the main species responsible for these changes (Miller *et al.*, 1964; Alm, 1982c).

2.3.3.3 *Utilization of proteins*

Milk proteins (caseins, whey proteins) are the main nitrogen source for lactic acid bacteria, which utilize them with the action of exocellular proteinases, membrane-bound aminopeptidases and intracellular exopeptidases and proteinases. Proteinase activity has been detected in several strains of lactobacilli and streptococci by Ezzat *et al.* (1985) and Kalantzopoulos *et al.* (1990). *L. delbrueckii* ssp. *bulgaricus* possesses a firmly cell-

bound proteinase with optimum activity between 45 and 50°C and pH values ranging from 5.2 to 5.8 (Argyle *et al.*, 1976). The partially purified cell-wall-associated proteinase studied by Ezzat *et al.* (1987) has maximum activity at 35°C and pH 5.5. The proteinase of *L. delbrueckii* ssp. *bulgaricus* is more active on β-casein than on whey proteins (Chandan *et al.*, 1982). El Soda and Desmazeaud (1982) and Laloi (1989) (In Zourari et al., 1992) revealed a preferential but partial hydrolysis of β- and α_{s1}-caseins. It was also observed that after growth in milk, caseinolytic activity was 3 times higher than that measured after growth in a complex medium rich in short peptides (Zourari et al., 1992).

Desmazeaud (1974) studied an intracellular metalloproteinase of *S. thermophilus* which was more active on the carboxy-methylated β-chain of insulin or on glucagons than on caseins. A weak caseinolytic activity was detected in both the cell envelopes and cytoplasm of *S. thermophilus*. As the two corresponding enzymes had the same electrophoretic mobility they were considered to be identical (Meyer *et al.*, 1989). A high cell-wall-associated proteinase activity characterizes 2 strains of *S. thermophilus* studied by Shahbal *et al.* (1991) who also obtained a proteinase-negative mutant. Strains which possess this proteolytic activity have a significantly higher acidification rate in milk compared with 13 other randomly chosen strains.

2.3.3.4 Utilization of peptides

The low molecular weight peptide fraction of milk is an important nitrogen source for yoghurt bacteria. The importance of peptides for their growth stimulation and their acidification is now well established, especially for *S. thermophilus*. *S. thermophilus* generally possesses a leucine-aminopeptidase activity. Some strains possess an arginine-amino-peptidase activity which is usually inactive against dipeptides. Two intracellular metalloenzymes of this species have been purified and characterized, i.e. an amino-peptidase with broad specificity and a dipeptidase which hydrolyzes dipeptides with a hydrophobic and bulky residue at the NH_2-terminal end (Rabier and Desmazeaud, 1973). A non specific prolyl-dipeptidase hydrolyzing several dipeptides has also been detected. An x-prolyl-dipeptidylamino-peptidase of a *S. thermophilus* strain has been purified,

characterized and compared with that of a *L. delbrueckii* ssp. *bulgaricus* strain. The streptococcal enzyme is composed of 2 subunits, has a molecular mass of 165 kDa, an isoelectric point of 4.5 and optimum pH values ranging from 6.5 to 8.2 (Meyer & Jordi, 1987). Two intracellular exopeptidases of *L. delbrueckii* ssp. *bulgaricus* have been characterized by El Soda and Desmazeaud (1982), an aminopeptidase with specificity limited to arginine-β-napthylamide and a dipeptidase with broad specificity (Zourari & Desmazeaud, 1992).

2.3.3.5 Digestion of milk proteins by yoghurt bacteria

Proteinase activity plays a role in the nutrition of lactic acid bacteria, since *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus* are auxotrophic for a number of amino acids. The principal substrate in milk for the proteolytic enzymes of yoghurt cultures is β-casein (Chandan *et al.*, 1982; Khalid *et al.*, 1991) although α_{s1}- (Ohmiya & Sato, 1969) and κ-casein (Khalid *et al.*, 1991) are degraded to a lesser extent. There have also been reports that whey proteins are degraded to a limited extent by these bacteria (Vaitheeswaran & Bhat, 1988).

Cell-wall-associated proteinases have been identified in *L. delbrueckii* ssp. *bulgaricus* (Ezzat *et al.*, 1985) as well in *S. thermophilus* (Shankar & Davies, 1978), although the activity of the former is much more important than the latter. It has been shown that the growth of *S. thermophilus* in milk is dramatically increased when casein hydrolysate is added (Thomas & Mills, 1981), indicating that proteinase activity of this culture is limiting for growth. While the growth of *L. delbrueckii* ssp. *bulgaricus* was also increased by addition of casein hydrolysate (Thomas & Mills, 1981), the increase was much less noticeable. Two strains of *S. thermophilus* with high proteinase activity have been isolated and their faster rate of acidification of milk has been directly correlated with their 7- to 10-fold increase in cell-wall-bound proteinase activity (Shahbal *et al.*, 1991). The growth of these strains was not stimulated by the addition of *Lactobacillus* extracts, contrary to most other strains of *S. thermophilus*. Cell-wall-associated proteinases from *L. delbrueckii* ssp. *bulgaricus* have also been isolated (Ezzat *et al.*, 1985; Laloi *et al.*, 1991; Argyle *et al.* 1976). Laloi *et al.* (1991) developed a method for

isolating cell-wall-associated protease activity, and the single proteinase activity isolated by them was purified to homogeneity in two steps. This enzyme hydrolyses both α- and β-caseins, albeit at different rates.

Peptidases have also been isolated from *L. delbrueckii* ssp. *bulgaricus* (Atlan *et al.*, 1989; Atlan *et al.*, 1990) and *S. thermophilus* (Casey & Meyer, 1985). The function of cell-wall-bound peptidases is to hydrolyse larger peptides into units no larger that four amino acids which can be transported through the cell membrane. Cytoplasmic peptidases subsequently hydrolyse the transported peptides. *L. delbrueckii* ssp *bulgaricus* has been shown to possess five aminopeptidase activities. Two APII and APIV, located at the cell surface, are constitutive, while two others, API, APII and X-Pro-DPAP, located in the cytoplasm, appear to be regulated (Atlan *et al.*, 1989, 1990). Intracellular peptidases have also been described for *S. thermophilus* (Rabier & Desmazeaud, 1974; Tsakalidou & Kalantzopoulos, 1992).

Some of the peptides resulting from milk protein degradation by yoghurt cultures have been characterized. Peptides resulting from the hydrolysis of β-casein by *L. delbrueckii* ssp. *bulgaricus* have been identified as being responsible for the bitterness in yoghurt. A "protein factor" resulting from the fermentation of milk by yoghurt cultures has been suggested as the factor responsible for the increased feed efficiency in rats fed yoghurt as compared to the control group which was fed milk. In addition, several bioactive peptides have been isolated from milk proteins. Casokinine, with antihypersensitive activity, originates from α- and β-casein, while casoplateline with anti-blood clotting activity originates in κ-casein. Casomorphine, a bioactive peptide with opioid activity, originates from α- and β-casein (Hartley & Denariaz, 1993).

2.3.4 Particular aspects of yoghurt fermentation and interactions between yoghurt bacteria

A positive interaction is generally observed between *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* in mixed culture, leading to the stimulation of growth and acid production of both bacteria compared to their single-strain cultures. In addition, total proteolysis in

mixed culture (expressed in μg of released tyrosine per ml of culture) exceeds the sum of the values obtained by each strain alone (Rajagobal & Sandine, 1990). Mixed yoghurt cultures may also stimulate the production of some metabolites such as acetaldehyde and influence carbohydrate utilization. For instance, one *L. delbrueckii* ssp. *bulgaricus* strain studied which cannot use galactose in pure culture metabolizes this sugar when it is associated with one strain of *S. thermophilus*. Contrary behaviour was observed with one streptococcal strain indicating that interactions between yoghurt bacteria are very complex and are greatly dependent on the strains involved (Zourari & Desmazeaud, 1992).

S. thermophilus does not possess substantial extracellular proteolytic activity and the amino acid and free peptide content of milk is not high enough to promote its full growth. Lactobacillus proteases break down caseins and supply the streptococci with amino acids and peptides. The growth of *L. delbrueckii* ssp. *bulgaricus* is stimulated by a compound produced by *S. thermophilus*, which appears to be formic acid (Shankar & Davies, 1978). Higashio *et al.* (1978) reported combined stimulating effect of formic and pyruvic acids. Zourari & Desmazeaud (1992) and Suzuki *et al.* (1986) observed that addition of formic acid to boiled milk prevents abnormal cell elongation in *L. delbrueckii* ssp *bulgaricus* (filamentous forms). Formic acid synthesis from pyruvate is a limiting step in purine synthesis and this explains the combined action of the 2 acids. When formate is lacking, ribonucleic acid (RNA) synthesis is depressed. Elongated cells contain less RNA than normal cells while DNA contents of both cells are almost equal. The combined growth of *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus* has the same effect as the addition of formic acid (Zourari & Desmazeaud, 1992).

Driessen *et al.* (1982) observed that stimulation of lactobacilli growth could also result from an increased CO_2 content in the growth medium, during continuous culture at constant pH. *S. thermophilus* produces a large quantity of CO_2 from milk urea. In this way, it can stimulate lactobacilli, since the quantity of CO_2 dissolved in milk decreases after heat treatment and so remaining CO_2 is too low to meet the requirements of the *Lactobacillus*. Carbon dioxide might be involved in the synthesis of aspartic acid. In

conclusion, interaction between yoghurt bacteria is a good example of integrated metabolism in a mixed culture of lactic acid bacteria, but our knowledge on the stimulation of *L. delbrueckii* ssp. *bulgaricus* by *S. thermophilus* is still incomplete (Zourari & Desmazeaud, 1992).

2.3.4.1 Production of polysaccharides

Bacteria synthesize a number of polysaccharides which are defined by their location relative to the cell. Some are intracellularly located in the cytosol and used as carbon sources, others are cell wall constituents such as peptidoglycan and teichoic acids and a third group is located outside the cell wall. The latter group can take the form of an adherent, often covalently bound, cohesive layer forming a morphological entity termed capsule or capsular polysaccharide (CPS). Alternatively, the polymer can consist of a slime polysaccharide with little or no cell association or attachment to the cell surface. In some cases, both capsular and unattached polysaccharides are produced by the same organism and distinguishing between the two forms can be difficult. Depending on their structural relationship to the bacterial cell, these polymers have been variously named slime, capsular or microcapsular polysaccharides. The term exopolysaccharide (EPS) as proposed by Sutherland (1972) provides a general name for all these forms of bacterial polysaccharides found outside the cell wall. In contrast to leuconostocs and oral streptococci, which produce homopolysaccharides, composed of a single sugar (dextrans, α-glucans, fructans), dairy lactic acid bacteria and propionibacteria produce heteropolysaccharides, composed of repeating units varying in size from disaccharides to heptasaccharides (Cerning, 1995).

2.3.4.1.1 Classification and characterization of exopolysaccharides from lactic acid bacteria

EPS from LAB can be subdivided into two groups: (1) homopolysaccharides, consisting of four subgroups, namely (a) α-D-glucans, i.e. dextrans and fructans, mainly composed of β-2,6-linked D-fructose molecules, such as levan with some β-2,1-branching through the O1 site; others, like polygalactan, composed of structurally identical repeating units with different glycosidic linkages; and (2) heteropolysaccharides produced by mesophilic

(*Lactococcus lactis* ssp. *lactis*, *L. lactis* ssp. *cremoris*, *Lactobacillus casei*, *L. sake*, *L. rhamnosus*, etc.) and thermophilic (*L. acidophilus*, *L. delbrueckii* ssp. *bulgaricus*, *L. helveticus* and *S. thermophilus*) LAB strains. The latter group of EPS receives renewed interest, since they play an important role in the rheology, texture and body, and mouthfeel of fermented milk drinks. For instance, the creamy, smooth texture is one of the aspects of the quality of yoghurt which seems to be improved by the ability of the yoghurt bacteria to produce EPS, even though small amounts of EPS are being produced (De Vuyst & Degeest, 1999).

Several gram-negative and gram-positive bacteria, including lactic acid bacteria, produce exocellular polysaccharides. The "ropy" character is often required for the manufacture of many fermented milk products. Ropy strains of lactococci have been isolated from Scandinavian fermented dairy products (Nakajima *et al.*, 1990). In stirred yoghurt, beverages and low milk solids yoghurts, production of polysaccharides can improve viscosity and texture, increase resistance to mechanical handling and decrease susceptibility to syneresis. The use of ropy strains (Figure 2.7) is particularly important in France and in the Netherlands where the addition of stabilizers in yoghurt is prohibited. In fact, some strains of *S. thermophilus* and *L. delbrueckii* ssp *bulgaricus* produce neutral exopolysaccharides. Only few studies are available on the characterization and production of these polymers (Cerning, 1990).

Schellhaass (1983) isolated ropy strains of some thermophilic lactic acid bacteria. She observed higher exopolysaccharide production in milk at suboptimal growth temperatures, as measured by an increase of relative viscosity. The polymer material obtained contained galactose and glucose in the ratio 2:1. The slime secreted by a strain of *L. delbrueckii* ssp. *bulgaricus* studied by Groux (1973b) contained mainly galactose and some glucose, mannose and arabinose. Cerning *et al.* (1988) studied an exocellular polysaccharide produced by *S. thermophilus*, which contains galactose (55%), glucose (25%), mannose (15%) and small amounts of rhamnose, xylose and arabinose. The *L. delbrueckii* ssp. *bulgaricus* exopolysaccharide studied by Cerning *et al.* (1986) contains galactose, glucose and rhamnose in an approximate molar ratio of 4:1:1 with a molecular

mass of about 500kDa and intrinsic viscosity of 4.7 dl/g. Doco *et al.* (1990) studied an exopolysaccharide produced by *S. thermophilus* after 3-4 h of incubation and obtained a polymer composed of galactose, glucose and N-acetylgalactosamine in a ratio of 2:1:1. The molecular mass of the polymer is 1×10^3 kDa and its intrinsic viscosity 1.54dl/g. Rhamnose, present mostly in the exopolysaccharide of *L. delbrueckii* ssp. *bulgaricus*, has not been identified in the exocellular polymer produced by 3 lactobacillus strains isolated from traditional Greek yoghurts (Zourari *et al.*, 1991). These molecules are composed of galactose (50-70%), glucose (20-40%) and some mannose and arabinose.

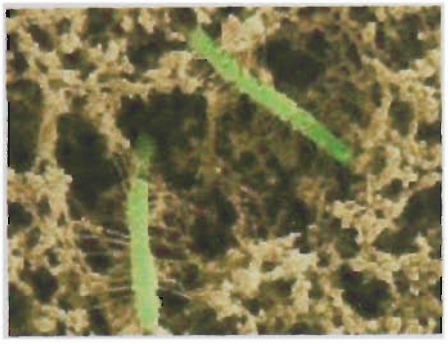

Figure 2.7 Ropy lactobacilli (Anon, 2000)

The quantities of polymer formed by ropy strains of both species vary considerably even under identical experimental conditions (Cerning *et al.*, 1990; Zourari *et al.*, 1991). It is difficult to establish a good correlation between the quantity of polysaccharide produced and the corresponding viscosity. This difficulty may be due to changes in the 3-dimensional configuration of polymers and to their interactions with some milk compounds, mainly caseins that are precipitated at low pH. In addition, viscosity measurements are difficult to interpret for non-Newtonian solutions such as milk and fermented milk products.

Bottazi and Bianchi (1986) studied the structure of milk fermented with a ropy *L. delbrueckii* ssp. *bulgaricus* strain by scanning electron microscopy (SEM). It was shown that a part of the polysaccharide covers the cells with a uniform layer, and the rest of the polymer binds cells together and to milk casein via a dense network of visible filaments. Recently, Teggatz and Morris (1990) used SEM to explain changes occurring in the

microstructure of ropy yoghurt when it is subjected to a shear force. Their aim was to understand the interactions with its surroundings, and how they may influence viscosity. It was observed that an increase of shear rate first disrupts the attachment of polymer to the bacterial surface, but the polysaccharide material remains incorporated with the casein where it continues to influence viscosity (Zourari & Desmazeaud, 1992).

The ropy character of *S. thermophilus* is often unstable (Cerning *et al.*, 1988). This may be partially due to the presence of glycohydrolase enzymes capable of hydrolyzing the polysaccharide material. There is no evidence of a possible loss of plasmids encoding this character as in *Lactococcus lactis* ssp. *cremoris* or in *Lactobacillus casei* ssp. *casei*. In fact, ropy strains of yoghurt bacteria studied by Cerning *et al.* (1986, 1988, and 1990) and Zourari (1991) are plasmid-free.

2.3.4.1.2 Nutrients enhancing growth and exopolysaccharide production of lactic acid bacteria

Enhanced EPS production and growth were initially obtained when (hydrolyzed) casein was added to skim milk cultures of *L. delbrueckii* ssp. *bulgaricus* (Cerning *et al.*, 1990). According to early investigations, neither growth nor EPS production was specifically linked to the presence of casein or whey proteins in the growth medium of LAB. However, Cerning *et al.* (1990) found that casein stimulates EPS production, but not growth of *L. delbrueckii* ssp. *bulgaricus*. On the other hand, addition of hydrolyzed casein to MRS did not increase specific polymer production by *L. delbrueckii* ssp. *bulgaricus* (De Vuyst & Degeest, 1999).

It has also been reported that *L. delbrueckii* ssp. *bulgaricus* is able to produce the same amount of EPS in milk and milk ultrafiltrate, but that *S. thermophilus* is not (Cerning *et al.*, 1990). On the other hand, supplementation of milk and milk ultrafiltrate with glucose or sucrose stimulates EPS production by *L. casei* and even modifies the monosaccharide composition of the EPS with glucose becoming dominant (De Vuyst & Degeest, 1999).

2.3.4.1.3 Application of slime-producing starter cultures

An alternative way to improve yoghurt viscosity and decrease susceptibility to syneresis and graininess is by utilizing the slime-producing strains in the starter culture. The ropy strains may further contribute to the consistency of stirred-type yoghurt, produced on a large scale, because yoghurt containing viscosifying EPS is supposed to be less damaged mechanically from pumping, blending and filling machines. In addition, the coagulum would be more resistant to thermal and physical shocks. This ultimately leads to the manufacture of yoghurt without the addition of stabilizers. This type of production process gains increased popularity in the Western world, because of the increased desire of the consumer for 100% natural products. Finally, EPS may play an important role in the production of other yoghurt drinks and low-milk-solid yoghurts, as well as in the production of creamier yoghurts with low or no fat content and an enhanced smoothness of mouthfeel. Because these products require increasing thickening properties, the use of slime-producing starter cultures in their manufacture is necessary (De Vuyst & Degeest, 1999).

The consistent manufacture of good quality yoghurts that have good texture, 'mouth-feel' and stability is important to the dairy industry. European yoghurt manufacturers have used 'texture-promoting' or 'ropy' cultures for many years, particularly where addition of stabilizers is prohibited. Even in countries where such addition is possible there is reluctance to jeopardize the 'natural' and 'additive-free' position of the products, with the result that the there is an increased interest in the texture-promoting qualities of these dairy strains. Cultures that are described as 'ropy' and used for their texture-enhancing properties are strains known to secrete exopolysaccharides (EPS), although little work has been published which relates the nature of the EPS to function, nor has there been much work on the related sensory perception of texture to instrumental data.

When broken and mechanically processed, yoghurt gels maintain a structure in which whey is largely retained. The use of EPS-producing strains in yoghurt-making can prevent syneresis (wheying off) and gel fracture (Cerning *et al.*, 1990), can increase viscosity and influence gel strength (Hess *et al.*, 1997). The quantities of EPS produced

by the dairy strains vary considerably. For example, the amounts of EPS from *S. thermophilus* can range from as little as 30 mg/l (Cerning *et al.*, 1988) to 890 mg/l (Escalante *et al.*, 1998), while for strains of *L. delbrueckii* ssp. *bulgaricus* it can range from 60 mg/l to 150 mg/l (Cerning *et al.*, 1986). Amounts and yields of EPS are also affected by associative growth. For example, Bouzar *et al.* (1997) showed that mixed cultures of a non-ropy strain of *S. thermophilus* and different ropy strains of *L. delbrueckii* ssp. *bulgaricus* produced EPS at a faster rate, while Rawson and Marshall (1997) found that the characteristics of texture enhancement and viscosity when mixing two ropy strains of these organisms together were not always additive (Marshall & Rawson, 1999).

Previous studies have shown that the sol-gel transitions of set-style yoghurts that occurred as a result of small deformations applied systematically to the gels during fermentation with ropy and non-ropy strains. It was also found that increasing the protein content of the base milk resulted in a less viscous product and that inclusion of a ropy strain had no effect on viscosity; however, culture differences were found which may suggest different effects due to different EPS. This was supported by other studies, where data collected pointed to the existence of a permanent network where protein strand formation and protein-protein bond development is partially prevented by the presence of polysaccharide. Further work on yoghurt gels suggested that the EPS-producing strains resulted in gels which had protein-polysaccharide bonds that had shorter relaxation times that protein-protein bonds and that attachment of mucogenic (ropy) strains to the protein matrix via EPS decreases yoghurt firmness (Marshall & Rawson, 1999).

Single point measurements such as gel strength have been used to characterize the mechanical behaviour of yoghurt gels. Such measurements, however, are based on rupture tests and may not be applicable to stirred yoghurt. Texture profile analysis (TPA) has been used as a technique for evaluating set yoghurt gels. TPA has also been used to examine the contribution of EPS from mesophilic lactococci to the texture of the Nordic fermented milk 'Viili', a visco-elastic product with unusual pouring consistency

(Marshall & Rawson, 1999). In a recent paper (Hess *et al.*, 1997) the TA-XT2 provided some further information on the nature of stirred yoghurt prepared using different thermophilic cultures. Techniques of texture measurements using a texture analyser and texture profile analysis attempt to imitate the conditions in the mouth to which the food is subjected (Marshall & Rawson, 1999).

Based on data obtained using a TA-XT2 texture analyser and TPA and from viscosity measurements, it has been shown that stirred yoghurt made with different EPS-producing thermophilic strains exhibited increased viscosity, an ability to recover viscosity lost as a result of mechanical disruption and an 'adhesiveness'. Although the characteristics observed were culture-dependent, it appeared likely that EPS was contributing to adhesiveness while 'firmness' and 'elasticity' were due to formation of a three-dimensional milk protein matrix (Marshall & Rawson, 1977).

2.3.4.2 Production of antimicrobial compounds by yoghurt bacteria

As mentioned above, there is generally a symbiotic relationship between yoghurt bacteria, but growth inhibition is sometimes observed. This should be taken into account when selecting starters. Inhibition may be due to competition for one or more nutrients of the growth or to inhibitory compounds produced by the strains, such as bacteriocins and inhibitory peptides (Zourari & Desmazeaud., 1992). Pulusani *et al.* (1979) (cited in Zourari *et al.*, 1992) extracted at least 3 fractions of ≈ 700 Da from milk cultured with *S. thermophilus*, which inhibited the growth of *Pseudomonas*, *Bacillus*, *E. coli*, *Flavobacterium*, *Shigella*, *Salmonella* and *Lactococcus* strains. These fractions are most likely aromatic amines released in the growth medium since cells are free of any antimicrobial activity. Glucose and lactose, but not sucrose, are essential for production of these compounds which are also obtained in milk-free media, e.g. "soy-milk" (Zourari & Desmazeaud, 1992).

Previous studies have shown that two bacteriocins of 10-20kDa produced by 2 *S. thermophilus* strains inhibited the growth of strains of the same species and to a lesser extent of enterobacteria. These molecules are sensitive to several proteases and to a

lipase, suggesting that a lipid component participates in their active sites. An antimicrobial substance named "bulgarican" produced by an *L. delbrueckii* ssp. *bulgaricus* strain has been partially purified. At neutral or acid pH, this heat-stable substance was active against several strains of *Bacillus*, *Streptococcus*, *Staphylococcus*, *Sarcina*, *Pseudomonas*, *Escherichia* and *Serratia* species (Zourari *et al.*, 1992).

L. acidophilus and *Bifidobacterium* are found naturally in the human intestines. The former culture dominates in the small intestine whereas the latter one in the large intestine. The growth of these bacteria has been reduced in some people as a result of consumption of different medical preparations, because of stress and from causes which have not yet been clearly defined. The reduced production of these important intestinal flora causes, in many people symptoms varying from swollenness to pronounced digestion problems. The consumption of live acidophilus and *Bifidobacterium* in milk products is an ideal way to contribute to reestablish the balance in the intestinal flora and prevent growth of harmful gram negative bacteria.

2.4 Nutritional and health values of fermented milk products

2.4.1 Historical background

Nutritional and health aspects of functional foods incorporating probiotic bacteria have received considerable attention, and eventually led to numerous purported claims in the literature. These potential advantageous properties, recently reviewed by Gilliland (1990) are summarized in Table 2.8. Despite the many studies on the beneficial nutropharmaceutical aspects of probiotic bacteria, the results obtained are highly variable and sometimes inconsistent with one another; hence, no clear, unequivocal evidence for the actual existence of some of these benefits is yet available, which then renders health claims more difficult to establish. To eliminate these drawbacks, efforts have been pursued worldwide to rationally organize future research using clearer and more reliable bases (via well-designed, randomized and placebo-controlled, double-blind studies) and exploiting statistically validated methods, with special emphasis on intestinal integrity

and immune modulation. Increasing numbers of colonization and dose-response studies defining the required doses have been published (Saxelin *et al.*, 1995).

Table 2.8 Potential health and nutritional benefits of functional foods prepared with probiotic bacteria[a]

Beneficial effect	Possible causes and mechanisms
Improved digestibility	Partial breakdown of proteins, fats and carbohydrates
Improved nutritional value	Higher levels of B vitamins and certain free amino acids, viz. methionine, lysine and tryptophan
Improved lactose utilization	Reduced lactose in product and further availability of lactase
Antagonistic action towards enteric pathogens	Disorders, such as functional diarrhoea, mucous colitis, ulcerated colitis, diverticulitis and antibiotic colitis controlled by acidity, microbial inhibitors and prevention of pathogen adhesion or pathogen activation
Colonisation in gut	Survival in gastric acid, resistance to lysozyme and low surface tension of intestine, adherence to intestinal mucosa, multiplication in the intestinal tract, immune modulation
Anticarcinogenic effect	Conversion of potential pre-carcinogens into less harmful compounds Inhibitory action towards some types of cancer, in particular cancers of the gastrointestinal tract by degradation of pre-carcinogens, reduction of carcinogen-promoting enzymes and stimulation of the immune system.
Hypocholesterolemic action	Production of inhibitors of cholesterol synthesis. Use of cholesterol by assimilation and precipitation with deconjugated bile salts
Immune modulation	Enhancement of macrophage formation, stimulation of production of suppressor cells and γ-interferon
Vaccine vehicle	Naturally occurring or rDNA vaccinal epitopes

[a] Adapted from Gomes & Malcata (1999)

2.4.2 Nutritional value

The nutritional benefits of probiotics have been mostly studied in milk-based products fermented with lactobacilli and bifidobacteria. These products contain a great many chemical compounds depending on the type of milk used (usually cow's, ewe's or goat's), on the type of microorganisms added (and their specific biochemical activities) and on the manufacturing processes employed. They are characterized by a lower level of residual lactose and higher levels of free amino acids and certain vitamins than non-fermented milks. Furthermore, they preferentially contain L (+)-lactic acid (that is more

easily metabolized by human beings than D (-)-lactic acid) produced by bifidobacteria in addition to acetic acid (Rasic & Kurmann, 1983), thereby preventing manifestation of metabolic acidosis in infants below one year of age. Moreover, the L (+)-lactic acid absorbed in the intestine is used as energy source, with an energy yield of 15kJ/g that compares well with 16kJ/g for lactose (Gomes *et al.*, 1999).

Lactobacillus acidophilus and bifidobacteria have also been reported to synthesize folic acid, niacin, thiamine, riboflavin, pyridoxine and vitamin K, which are slowly absorbed by the body (Rasic & Kurmann, 1983; Tamime *et al.*, 1995). The vitamins of the B-complex are frequently obtained as natural ingredients in foods, so addition of bifidobacteria to the diet will more effectively help meet those requirements. The bioavailability to the host of such minerals as calcium, zinc, iron, manganese, copper and phosphorous may also be enhanced upon consumption of fermented dairy products containing bifidobacteria via lowering the gastric pH (which facilitates ionization of minerals, a requirement for absorption) and improved digestibility of the protein (Rasic & Kurmann, 1983).

2.5 Therapeutic potential and health benefits of *Lactobacillus acidophilus* and bifidobacteria

The main therapeutic and health benefits of *L. acidophilus* and bifidobacteria are: (i) enhancement of immunity against intestinal infections; (ii) immune enhancement; (iii) prevention of diarrhoeal diseases; (iv) prevention of colon cancer; (v) prevention of hypercholesterolaemia; (vi) improvement in lactose utilization; (vii) prevention of upper gastrointestinal tract diseases; and (viii) stabilization of the gut mucosal barrier (Kailasapathy and Chin, 2000).

The major therapeutic considerations of both bifidobacteria and *L. acidophilus* discussed here are (a) antimicrobial activity, (b) anticarcinogenic activity, (c) cholesterol reduction, (d) alleviation of lactose intolerance, and (e) vitamin synthesis.

(a) Antimicrobial activity: Both bifidobacteria and *L. acidophilus* show antagonistic effects towards enteropathogenic *E. coli*, *Salmonella typhimurium*, *Staphylococcus aureus* and *Clostridium perfringens* (Gilliland & Speck, 1977). *L. acidophilus* produces various antibacterial substances such as Lactocidin, Acidolin, Acipophilin, Lactacium-B and inhibitory protein. The strains of *Bifidobacterium* produce Bifidolin and Bifilong which inhibit several pathogenic bacteria. The production of hydrogen peroxide, which is inhibitory to many pathogens by *L. acidophilus*, was also observed. Bifidobacteria are capable of correcting abnormal adult microflora which cause serious adverse consequences (Vijayendra & Gupta, 1992).

(b) Anticarcinogenic activity: The lactic acid bacteria and dairy products fermented by them have shown anticarcinogenic activity. It is due to the compounds produced by the organisms during growth. The anticarcinogenic effect of bifidobacteria may be the result of direct or indirect removal of procarcinogens or by the activation of the immune system (Fernandes & Shahani, 1990). *Bifidobacterium* demonstrates a high absorbing property for carcinogenics that are produced upon the charring of meats (Mitsuoka, 1989). Indirectly bifidobacteria remove the source of procarcinogens or the enzymes which lead to their formation (Vijayendra & Gupta, 1992; Lankaputhra & Shah, 1998).

(c) Reduction of cholesterol level: There is a high correlation between dietary saturated fat or cholesterol intake and serum cholesterol level. The level of serum cholesterol is a major factor of coronary heart diseases. Previous studies have observed a lower serum cholesterol concentration in rats fed with fermented milk containing *L. acidophilus* than did the water fed or milk fed rats. Kim (1988) ascribed the hypocholesterolemic effect to lactobacilli. The lowering of serum cholesterol level in rats fed orally with bifidobacteria has also been reported. This effect might be due to the inhibition of the hydroxymethyl glutaryl CoA reductase, the regulatory enzyme in cholesterol synthesis (Vijayendra & Gupta, 1992).

(d) Alleviation of lactose intolerance: Lactose maldigestion is the result of insufficient amounts of β-galactosidase in the human small intestine to digest the milk sugar.

Individuals with this condition experience gastric distress when they consume fresh unfermented dairy products, due to the formation of hydrogen gas by microbial action on undigested lactose in the gut (Gilliland, 1989). β-Galactosidase, the enzyme necessary for lactose digestion is affected by bile. Because bifidobacteria are resistant to bile, they may have a better chance of colonizing the gut and delivering their metabolizing enzyme to its site of action over an extended period of time (Vijayendra & Gupta, 1992).

(e) Vitamin synthesis: Bifidobacteria are known to produce thiamine, riboflavin, folic acid and vitamins B1, B6 and B12. Bifidus fermented milk products may be a good source of B-complex vitamins (Vijayendra & Gupta, 1992).

2.6 Viability and survival of *Lactobacillus acidophilus* and bifidobacteria

The contribution of yoghurt bacteria to the improvement of intestinal microflora has been widely recognized. The incorporation of *L. acidophilus* and *B. bifidum* into the yoghurt starter culture produces a milk product of excellent 'therapeutic' value (Tamime & Robinson, 1985). Regular consumption of yoghurt (400-500 g/week) containing 1.0×10^6 CFU/g of *Bifidobacterium* ssp. and *L. acidophilus* (human origin), which are able to survive the upper regions of the gastrointestinal tract, is essential to achieve therapeutic benefits (Tamime *et al.*, 1995).

Viability and activity of the bacteria are important considerations, because the bacteria must survive in the food during shelf life and during transit through the acidic conditions of the stomach, and resist degradation by hydrolytic enzymes and bile salts in the small intestine (Playne, 1994). It is essential that products sold with any health claims meet the criterion of a minimum 10^6 CFU/mL probiotic bacteria at the expiry date, because the minimum therapeutic dose per day is suggested to be 10^8-10^9 cells (Kurmann & Rasic, 1991). The range of yoghurts and dried yoghurt preparations available in Australia that contain probiotics is large. However, evaluation by the Dairy Research Laboratory, CSIRO (Highett, Vic., Australia) and others (Anon, 1992) showed that the probiotic

organisms are often not at high levels and that their activity may not be optimal (Anon, 1992).

Strains of bifidobacteria used in some commercial products survive neither gastric transit nor product acidity during storage (Varnam & Sutherland, 1994). In a previous study, five brands of Australian commercial yoghurts obtained directly from processors were enumerated for viable *L. acidophilus* and *B. bifidum* at 3 day intervals for 5 weeks (Shah *et al.*, 1995;Dave & Shah, 1997; Shah *et al.*, 2000). Three of the five brands contained 10^7-10^8 viable cells of *L. acidophilus* per gram whereas the other two products contained $<10^5$/g of this organism initially. The initial count of *B. bifidum* was 10^6-10^7/g in two of the five products, while the viable numbers of this organism were $<10^3$/g in the other three products. All the products showed a constant decline in the viable count of *L. acidophilus* and *B. bifidum* during storage. It is misleading to describe probiotic yoghurt as having health promoting properties unless the minimum level of viable cells is present at the expiry date (Kailasapathy & Chin, 2000).

In Japan, bifidobacteria containing products are very popular and these products account for more than one-third of the total yoghurt sales. In France, products containing bifidobacteria and *L. acidophilus* have increased by ~300% to capture 4% of total fresh milk sales (Hughes and Hoover, 1991). Presently, 11% of all yoghurt sold in France contains added bifidobacteria. In Germany, one of the first bifidus products to be marketed, known as Biogarde, sold more than 400 million units in 1976 and the product is well established in the market. Biogarde is produced by 45 dairy companies in Germany. In Denmark, a product called Cultura was promoted as a completely safe and easily digestible food. Bifidus products are also produced in Canada, Italy, Poland, the United Kingdom, Czechoslovakia, and Brazil. In the United States and Australia, bifidus products have been introduced in recent years (Hughes and Hoover, 1991).

In Europe and Australia, yoghurt containing *L. acidophilus* and *Bifidobacterium* ssp. is referred to as 'AB' yoghurt. The trend is to incorporate *L. casei* in addition to *L. acidophilus* and bifidobacteria and such products are known as 'ABC' yoghurt.

Traditionally, yoghurt is manufactured using *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* as starter cultures. These yoghurt organisms are claimed to offer some health benefits; however, they are not natural inhabitants of the intestine. Therefore, for yoghurt to be considered as a probiotic product, *L. acidophilus* and bifidobacteria (and *L. casei* or both) are incorporated as dietary adjuncts. Products such as yakult contains the *L. acidophilus* Shirota strain. Thus, fermented milk with only *L. acidophilus* or *L. acidophilus* and *Bifidobacterium* ssp. could be manufactured; however, the longer incubation period and product quality are the two main factors that are sacrificed when fermenting milk with only 'AB' or 'ABC' bacteria. Thus, the normal practice is to make products with both yoghurt and probiotic bacteria.

According to the Australian Food Standards Code (Standard H8), yoghurt must have a pH of $\leq$4.5 and must be prepared with *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* or other suitable lactic acid bacteria. The Australian Food Standards Code does not specify any requirements regarding the numbers of yoghurt or probiotic bacteria in the fermented products. However, in other countries standards have been developed regarding the requirement of the numbers of the probiotic bacteria in fermented products. In Japan, a standard has been developed by the Fermented Milks and Lactic Acid Bacteria Association, which requires a minimum of 10^7 viable probiotic bacteria cells per milliliter to be present in fresh dairy products (Robinson, 1987). Therefore, efforts to select the right type of strain and improvement in viability are commercially significant. Beneficial and proven strains could be obtained through a reputed starter culture supplier; however, the viability of organisms during manufacture and storage is the sole responsibility of the manufacturers. Several reports have shown that the viability of these organisms is often low in yoghurt (Gilliland & Speck, 1977; Hull *et al.*, 1984; Schioppa *et al.*, 1981). A number of brands of commercial yoghurts have been analyzed in Australia (Anon, 1992; Shah *et al.*, 1995, 2000) and in Europe (Iwana *et al.*, 1993) for the presence of *L. acidophilus* and bifidobacteria. Most of the products contained very low numbers of these organisms, especially bifidobacteria.

The viability of probiotic bacteria is affected by inhibitory substances such as lactic acid produced during production and cold storage. During production of yoghurt, yoghurt bacteria and probiotic bacteria produce organic acids. The pH of the product needs to be 4.5 or lower to meet legal requirements and to produce good quality yoghurt. Also, depending on the extent of growth of bifidobacteria, concentration of acetic acid could vary in the product.

Several factors have been claimed to affect the viability of yoghurt and probiotic cultures in fermented milk products. The viability of probiotic bacteria in yoghurt depends on the strains used, interaction between species present, culture conditions, production of hydrogen peroxide due to bacterial metabolism, final acidity of the product, and the concentrations of lactic and acetic acids. The viability also depends on the availability of nutrients, growth promoters and inhibitors, concentration of sugars (osmotic pressure), dissolved oxygen and oxygen permeation through package (especially for *Bifidobacterium* ssp.), inoculation level, incubation temperature, fermentation time and storage temperature (Bertoni, 1994; Costello, 1993; Gilliland & Lara, 1988; Young & Nelson, 1978). However, the main factors for loss of viability of probiotic organisms have been attributed to a decrease in the pH of the medium and accumulation of organic acids as a result of growth and fermentation (Hood & Zottola, 1988; Shah & Jelen, 1990). Bifidobacteria are anaerobic in nature; therefore, higher oxygen content may affect their growth and viability. The availability of growth factors is also reported to affect the growth and viability of yoghurt and probiotic bacteria. Antagonism among the bacteria used in the starter cultures caused by the production of antimicrobial substances such as bacteriocins may decrease the numbers of any sensitive organisms that may be present in a product or starter cultures (Shah, 2000).

L. acidophilus and *B. bifidum* are inhabitants of the intestines of humans as well as animals. Beneficial effects of *L. acidophilus* and *Bifidobacterium* ssp. can be expected only when viable cells of these organisms are able to survive passage through the human stomach and digestive system and colonize in the human gut. One of the requirements for micro-organisms to be used as dietary adjuncts is the need to retain viability and

activity in the food vehicle before consumption (Kim, 1998). Buffering capacity of yoghurt provides protection for micro-organisms in the gastrointestinal tract.

The bacterial strains that are selected for probiotic milk products by the culture supplier should fulfill the following requirements: survive the gastric acidic conditions (pH 1-4), be resistant to the action of bile salts, resist degradation by digestive enzymes present in the intestines (e.g. lysozymes), survive action of toxic metabolites, primarily phenols, produced during the digestion process, antibiotics and phage, anaerobic growth conditions and storage conditions of the food carrier.

Bifidobacteria may help to stabilize the digestive systems of human beings; however, it is essential that: (i) the carrier food contains not less than 1 million viable cells of *Bifidobacterium*/gram of a product; (ii) the species is of human origin (e.g. *B. bifidum*, *B. longum*, *B. adolescentis* or *B. infantis*); and (iii) the total intake per week per product is approximately 300-400g (Samona & Robinson, 1994).

Lactobacillus delbrueckii ssp. *bulgaricus* and *S. thermophilus* (yoghurt starter cultures) are not bile resistant and do not survive the passage through the intestinal tract (Gilliland, 1978). However, *L. acidophilus* and *B. bifidum* incorporated into the yoghurt starter culture have the ability to establish themselves among the gut flora (Tamime & Robinson, 1985). Growth of bifidobacteria is adversely influenced by the presence of yoghurt cultures (Samona & Robinson, 1994). Strains of *Bifidobacterium* used in some commercial products do not survive gastric transit nor product acidity during storage (Varnam & Sutherland, 1994). While the presence of yoghurt cultures may restrict the growth of bifidobacteria, they have comparatively little impact on the long-term viability of an existing culture. Thus, as long as an acidophilus-bifidobacter (AB) yoghurt manufacturer ensures at least 1 million viable cells of *Bifidobacterium* ssp. per gram at the end of incubation, the number of probiotic bacteria should remain stable throughout the anticipated shelf life (Samona & Robinson, 1994).

The stomach has the highest acidity and the pH of these areas may fall to as low as 1.5. *Lactobacillus acidophilus* and *Bifidobacterium* ssp. must be able to survive these adverse conditions in order to colonize in the gut. Many strains of *L. acidophilus* and *Bifidobacterium* ssp. intrinsically lack the ability to survive adverse conditions in the gut. Survival of *L. acidophilus* and *B. bifidum* depends on the pH of the environment; low pH decreases their survival. Under acidic conditions (both *in vitro* and *in vivo*), probiotic organisms, such as *L. acidophilus*, survive better that the traditional culture organisms (*L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus*) in yoghurt (Shah and Jelen, 1990). Hood and Zottola (1988) have reported that *L. acidophilus* (BG2FO4) shows rapid decline in numbers at pH 2.0, but at pH 4.0 the numbers of viable cells do not increase significantly (Hood and Zottola, 1988). These results have been confirmed by Lankaputhra and Shah (1995) who concluded that six strains of *L. acidophilus* studied survived well at pH 3.0 or above and the viable counts remained > 10^7 CFU/g after 3 h incubation. However, Playne (1993) has reported that *L. acidophilus* does not grow well below pH 4.0. Rius *et al* (1994) reported that *L. acidophilus* has high cytoplasmic buffering capacity (pH 3.72-7.74), which may allow it to resist changes in cytoplasmic pH and gain stability under acidic conditions. *Lactobacillus acidophilus* is more tolerant to acidic conditions than *B. bifidum* and the growth of *B. bifidum* is significantly retarded below pH 5.0 (Costello, 1993). However, tolerance of *Bifidobacterium* to acidic stomach conditions has been reported to be strain specific (Berada *et al.*, 1991). Clark *et al.* (1993) have reported that *B. longum* shows better survival in acidic conditions compared with *B. infantis*, *B. adolescentis* and *B. bifidum*. Lankaputhra and Shah (1995) have studied survival of nine strains of *Bifidobacterium* ssp. in acidic conditions (pH 1.5-3.0); *B. longum* and *B. pseudolongum* show the greatest survival. They have also reported that *B. adolescentis* and *B. breve* survive poorly at all levels (1.5, 2.0, 2.5 and 3.0) tested. Encapsulation of bacteria in milk fat does not improve the survival of bifidobacteria in high acid yoghurt (Modler *et al.*, 1993; Shah & Ravula, 2000).

Gastrointestinal systems have varying concentrations of bile. The rate of secretion of bile acid and its concentration depend on the type of food consumed. For example, the fat content of the diet could influence the level of faecal bile acids and fatty acids may

increase the inhibitory effects of bile acids towards *Lactobacillus* and *Bifidobacterium* ssp. (Alm, 1991). Bile concentrations range from 0.5 to 2.0% in the first hour of digestion and the levels may decrease during the second hour (Kailasapathy & Rybka, 1997). Ibrahim and Bezkorovainy (1993) have reported that bifidobacteria are able to survive physiological and higher bile salt levels. They have reported that at 0.06-0.03% sodium glycolate, *B. infantis* showed the best survival, followed by *B. bifidum* and *B. breve*, whereas *B. longum* showed least resistance to bile. However, Clark and Martin (1994) have reported that *B. longum* shows the best survival rates in 2% and 4% bile. Survival numbers are approximately 10^7 CFU/mL after 12 h in 2% bile. Lankaputhra and Shah (1996) have reported that among six strains of lactobacilli, two strains (*L. acidophilus* 2404 and 2415) showed the best tolerance to bile (1-1.5%) and among nine strains of *Bifidobacterium* ssp., *B. longum*, *B. pseudolongum* and *B. infantis* showed the best tolerance to bile (1-1.5%). They have also pointed out that while one strain of *B. longum* (1941) was tolerant to bile, the other strain of *B. longum* (20097) did not survive well in bile. Holcomb *et al.* (1991) have reported that both *L. acidophilus* and *Bifidobacterium* were able to survive and grow in soft-serve frozen yoghurt after freezing and both were found to grow in up to 0.45% bile salts, before and after freezing.

The presence of oxygen, specific growth-promoting factors and oxidation-reduction potential of the growth medium also affect the survival of bifidobacteria. Water activity is another critical factor in maintaining maximal survival of probiotic cultures over the shelf life required. It has been shown that, for maximum survival of *L. acidophilus* in freeze-dried type cultures when stored at room temperature, the water activity should be less than 0.25 Units (Sellars, 1991).

Lactic acid bacteria are susceptible to inhibition by bacteriocin or bacteriocin-like substances (Marshall, 1991). Hydrogen peroxide produced by *L. delbrueckii* ssp. *bulgaricus* was responsible for the loss in viability of *L. acidophilus* when added in yoghurt (Gilliland & Speck, 1997). According to these authors, it is not reasonable to expect that *L. acidophilus* could survive in yoghurt in sufficient numbers to influence the flora in the intestinal tract. Yoghurt also has been found to be an unacceptable vehicle to

incorporate *Bifidobacterium* in the human diet, due to the high acidity of most products (Modler & Villa-Garcia, 1993). However, survival of *L. acidophilus* is high when the organisms are added with the starter before manufacture (Hull *et al.* 1984). *Lactobacillus acidophilus* and *B. bifidum* grow well at low surface tension and are resistant to lysozyme (Gilliland, 1978), which would allow them to survive and grow in the intestinal tract. The adhesion mechanism of *L. acidophilus* is mediated by lectins, synthesized by lactobacilli or by epithelial cells of intestines (Kailasapathy & Chin, 2000)

2.7 Dairy products supplemented with *Bifidobacterium* ssp. and/or *Lactobacillus acidophilus*

The species most frequently employed in the production of probiotic dairy products are of human intestinal because it is generally accepted that they are better suited to the physiological needs of the host and can more easily colonize his/her intestine than wild strains, or strains that exist in the colon of other animals. These human-borne strains include *Bifidobacterium adolescentis*, *B. bifidum*, *B. breve*, *B. infantis*, *B. longum*, *Lactobacillus acidophilus*, *L. casei* ssp. *rhamnosus* and *Enterococcus faecium* (Kurmann, 1998; Hoier, 1992; La Torre *et al.*, 2003). Results of recent studies support the use of selected strains of *B. longum* as dietary adjuncts in dairy products, with *B. adolescentis* and *B. infantis* as adequate alternatives (Martin, 1996). It is again worth noting that each strain within these species exhibits unique properties, growth rate, metabolic rate, proteolytic activity and flavour promotion. Consequently, careful management of such factors via tailored manufacturing technologies will enable these species to meet the varying degrees of success in multiple industrial applications. Cases of milk products fermented by bifidobacteria of animal origin have also been reported these strains are much easier to cultivate and can withstand the adverse conditions encountered during industrial production, viz. low pH and presence of detectable levels of oxygen. In addition to a beneficial role after consumption (Gomes, 1999), the recently identified *B. lactis* is also a promising candidate due to its good acid and oxygen tolerance (Gomes *et al.*, 1998).

The most frequent functional food products in the market are of dairy origin (Table 2.9): Japan, a leading country in their manufacture, produces and markets more than 50

different dairy products containing viable cells. Similar trends are also observed in such developed European countries as France, Germany and Sweden (where probiotic products account for ~25% of all fermented milk products). It is estimated that there are ~80 bifido-containing products available in the world market, and more than 45 dairy plants in Europe currently produce bifido-acidophilus products (Hoier, 1992). Besides the commercial products, research studies have been more and more focused on fermented milks (Mital & Garg, 1992), yoghurt (Reuter, 1990), frozen yoghurt and desserts (Laroia & Martin, 1991; Modler & Villa-Garcia, 1993), ice cream (Modler *et al.*, 1990; Hekmat & McMahon, 1992), cheese (Dinakar & Mistry, 1994; Gomes *et al.*, 1995), fermented soya milk (Valdez & de Giori, 1993) and soya yoghurt (Murti *et al.*, 1992). The designation of new products is normally obtained via adding the prefix 'Bio' to its traditional (sometimes partially simplified) name (e.g. Biogurt, Biodrink, Biokys).

The probiotic strains of bifidobacteria and *L. acidophilus* may be added, either alone or following combination with other lactic acid bacteria during fermentation, to the final fermented product, or to the fresh product before distribution. General accounts of the physiochemical and technological aspects of commercial fermented milk products containing *Bifidobacterium* ssp. and *L. acidophilus* have been provided by Kurmann and Rasic (1991), Mital and Garg (1992) and Tamime *et al.* (1995). In addition to food products containing probiotic bacteria, there are various health products as well as pharmaceutical preparations containing probiotics, available in the market. In general, these consist in encapsulated freeze-dried bacterial populations that are used in the treatment of gastrointestinal disturbances (diarrhoea, including side effects of antibiotic therapy), constipation and certain hepatic diseases (Gomes *et al.*, 1999).

Table 2.9 Commercial products containing *Bifidobacterium* ssp. and *Lactobacillus acidophilus*[a]

Product	Country of origin	Microorganism
A-38	Denmark	*Lactobacillus acidophilus, Bifidobacterium bifidum, Leuconostoc mesenteroides* ssp. *cremoris*, mesophilic lactococci
Acidophilus buttermilk	USA	*Lactobacillus acidophilus, Leuconostoc mesenteroides* ssp. *cremoris*, mesophilic lactococci
Progurt		*Bifidobacterium bifidum, Lactobacillus acidophilus*, mesophilic lactococci
Acidophilus milk	Several countries	*Lactobacillus acidophilus*
Acidophilus yeast milk	Former USSR	*Lactobacillus acidophilus, Saccharomyces fragilis, S. cerevisiae*
A-B Yoghurt	France	*Bifidobacterium bifidum, Lactobacillus acidophilus*
Cultura	Denmark	Ibidem
Milky	Italy	Ibidem
Nu-Trish A/B Milk	USA	Ibedim
Biomild	Several countries	*Bifidobacterium ssp., Lactobacillus acidophilus*
Acidophilus yoghurt (ACO-yoghurt)	Several countries	*Lactobacillus acidophilus, L. delbrueckii ssp. bulgaricus, Streptococcus thermophilus*
B-Active	France	*Lactobacillus acidophilus, Bifidobacterium bifidum, L. delbrueckii* ssp. *bulgaricus, Streptococcus thermophilus*
Fresh BA	UK	Ibidem
Kyr	Italy	Ibidem
Yoplus	Australia	Ibidem
Vitalite	Australia	Acidophilus and Bifidus cultures (AB cultures),
Biogarde	Germany	*Lactobacillus acidophilus, Bifidobacterium bifidum, Streptococcus thermophilus*
Ofilus	France	Ibidem
Philus	Norway	Ibidem
Bifidus milk	Several countries	*Bifidobacterium bifidum, B. longum*
Bifighurt	Germany	*Bifidobacterium bifidum, Streptococcus thermophilus*
Biogurt	Germany	*Lactobacillus acidophilus, Streptococcus thermophilus*
Biokys	Czech Republic	*Bifidobacterium bifidum, Lactobacillus acidophilus, Pediococcus acidilactici*
Mil-Mil	Japan	*Lactobacillus acidophilus, Bifidobacterium bifidum, B. breve*
Yakult	Japan	*Lactobacillus acidophilus, Bifidobacterium bifidum, B. breve, L. casei* ssp. *casei*

[a] Adapted from Kurmann (1998) and Hoier (1992)

2.8 *Possible ways of increasing the survival of L. acidophilus and bifidobacteria*

There are a number of substances known to improve or promote the growth of probiotic bacteria. Tomato juice and papaya pulp stimulate the growth of *L. acidophilus*, resulting in higher viable counts, shorter generation times and improved sugar utilization (Babu *et al.*, 1992). The stimulation could be attributed to greater availability of simple sugars, mainly glucose and fructose, and minerals (i.e. magnesium and manganese), which are growth promoters for *L. acidophilus* (Ahmed & Mital, 1990). Supplementation of milk with a combination of casitone, casein hydrolysate and fructose results in a similar effect (Shimamura, 1982). Growth of *L. acidophilus* is enhanced by acetate (Marshall, 1991).

Bifidobacteria show poor growth in milk. Vitamins, dextrin and maltose stimulate their growth, but sucrose and iron salts have little effect. The survival of *B. longum* in milk can be improved by addition of 0.01% bakers yeast (Shimamura, 1982). Oligosaccharides allow the preferential growth of probiotic organisms in the colon, because these substances are not used by other intestinal bacteria (Anon, 1992). Most *Bifidobacterium* species metabolize a wide range of indigestible polysaccharides and oligosaccharides to acetic and lactic acids. Raffinose, stachyose, fructo-, isomalto- and galacto-oligosaccharides are effective for proliferation of resident or implanted bifidobacteria (Mitsuoka, 1992). Bifidobacteria also use lactulose preferentially in the colon as a source of energy (Rasic & Kurman, 1983).

Manipulating the conditions in the manufacture and storage of yoghurt could increase the survival of lactic acid bacteria and bifidobacteria. The following methods have been reported to achieve this objective:

1. Terminating fermentation at a higher pH (>5); allows better survival of bifidobacteria (Varnam & Sutherland, 1994).
2. Lowering the storage temperature to less than 3-4°C increases AB culture (*L. acidophilus* and bifidobacteria) survival (Sakai *et al.*, 1987).

3. Enrichment of yoghurt mix with whey protein concentrate (increases the buffering capacity of yoghurt, retards decrease in pH and prevents pH change during storage of yoghurt) (Supriadi *et al.*, 1994; Dave & Shah, 1998).
4. Application of hydrostatic pressure (200-300MPa for 10 min at room temperature) to yoghurt prevents after-acidification and hence maintains initial number of viable lactic acid bacteria (Tanaka & Hatanaka, 1992).
5. Heat shock (58°C for 5 min) of the yoghurt (prevents excess acid production and acidity remains constant during storage) (Marshall, 1992).
6. Lowering the incubation temperature to 37°C favours growth of bifidobacteria and increases incubation time (Costello, 1993).

Care should be exercised in the selection of culture organisms, because the composition and stability of the culture, size of inoculum, fermentation conditions and species interrelationships, have been reported to influence the survival of probiotic bacteria. The selection or development of acid-tolerant strains of AB culture to make them viable in low pH yoghurts is essential for enhanced survival of these organisms (Costello, 1993; Martin & Chou, 1992). For example, the variants of bifidobacteria obtained by Bresske *et al.* (1994) had an enhanced yield (by 20-25%) and acid-forming ability and inhibited a wider variety of pathogenic bacteria than control bifidobacteria. The cell count at the end of incubation must be sufficiently high to allow up to 90% mortality of probiotic bacteria during storage and still leave their number above the desired minimum of 10^6 CFU/mL viable cells (Marshall, 1991). The use of a concentrated deep-frozen or freeze-dried culture, which may be applied in the direct preparation of bulk starter, is recommended (Costello, 1993; Babu *et al.*, 1992). Usage of a high level of inoculum (up to 10-20%) is recommended (Varnam & Sutherland, 1994). Excluding *L. delbrueckii* ssp. *bulgaricus* from the fermentation will eliminate antagonistic effects of substances such as hydrogen peroxide towards AB cultures (Rybka, 1994). Beneficial interrelationships occur between *S. thermophilus* and *Bifidobacterium*, where the former organism acts as an oxygen scavenger and creates anaerobic conditions that could enhance the growth and survival of the latter organism (Rybka, 1994; Gilliland, 1991;

Shankar & Davies, 1976). Microencapsulation and coating of organisms have been used satisfactorily to increase their survival in human gastric and intestinal juices (Kailasapathy & Chin, 2000).

2.9 Milk composition

As in food regulations of many countries, "milk" without any further indication denotes cow's milk. Milk can be defined as being the normal lacteal secretion obtained from the mammary gland of the cow. Besides, water, normal cow's milk contains about 5% of milk sugar (lactose), about 3.2% milk protein, 3-5% or more milk fat depending on breed, type of feed, season, and other factors (a typical value for bulk delivered milk is 3.6-3.8% fat), and about 0.75% minerals. The principal constituents of these proximate component groups are listed in Table 2.10. Before processing, the total solids content of the fluid milk is about 13%, which is more than in many solid foods, especially fruits and vegetables (Jelen, 1985).

In the physicochemical sense, milk is a very interesting system simultaneously representing a true solution (salts and lactose in water), emulsion (milk fat globules in the water solution), as well as colloidal dispersion (protein particles). Casein, the major milk protein, is not truly soluble in water; in the milk system it is dispersed in small units called casein micelles which do not settle under normal gravitational conditions. This gives milk its whiteness, since the casein micelles, containing several hundred casein molecules, are large enough to scatter light (Jelen, 1985).

Table 2.10 Principal constituents of cows milk (average values).

Component group (content in milk, % w/w)	Main components	Proportion of components within component group %
Proteins (3.2)		
	Casein	80
	Whey proteins	15, of which
	α-lactalbumin	3
	β-lactoglobulin	9
	Other minor proteins	3
	Non-protein nitrogen	5
Fats (3.7)		
	Milk fat	≥ 98
	Fat globule membrane	≤ 2
Carbohydrates (4.9)		
	Lactose	> 99
	Glucose	< 1
	Galactose	Trace
Minerals and salt constituents (0.75)		
	Calcium	18
	Sodium	8
	Potassium	20
	Phosphorous	13
	Chlorine	16
	Other (magnesium, citrate, carbonate)	25

Source: Jelen (1985)

In addition to meeting the requirements for various nutrients, milk supplies:

- Protective factors, e.g. immunoglobulins, lactoferrin and some enzymes, e.g. lysozyme and lactoperoxidase;
- Growth factors and other hormones;
- Digestive/nutritional aids, e.g. vitamin-or metal-binding proteins and lipase.

It should be noted that these non-nutritional functions are performed by proteins that have attracted considerable attention as a means of increasing the biological and monetary value of dairy products but they are, in fact, trivial in terms of the total value of the dairy industry. A third group of dairy-derived neutraceuticals are peptides with biological

properties that are released from the milk proteins when subjected to proteolysis either *in vitro* or *in vivo*. These peptides have attracted considerable attention but most are still at the experimental stage and may not even function *in vivo* (Fox, 2001).

In addition to the proteins, lactose, fat and minerals, milk contains a wide variety of minor components such as vitamins, enzymes, trace elements and other compounds. Some of these play an important role in dairy processing and/or in human nutrition. Table 2.11 gives a partial list of some of the minor constituents significant for the dairy industry (Jelen, 1985).

Table 2.11 Minor constituents of cow's milk with importance to the dairy industry

Component group	Example	Importance to dairy industry
Enzymes	Lipase	Fat breakdown (rancidity) in improperly processed products
	Alkaline phosphatase	Indicator of properly pasteurization treatment
	Protease (also of microbial origin)	Bitterness in cheese and other products
Vitamins	Vitamin A + carotenoids	Nutritional importance (good source); yellow colour of butterfat
	Riboflavin (vitamin B2)	Nutritional importance (excellent source); light sensitive; greenish color of cheese whey
	Vitamin D	Nutritional importance for dietary absorption of calcium; added since milk contains little
Trace elements	Copper	Catalyzes undesirable oxidation of milk fat; some nutritional importance
	Zinc	Nutritional importance

Source: Jelen (1985).

The proteins of milk belong to two main categories, which can be separated based on their solubility at pH 4.6, 20°C. Under these conditions caseins precipitates. The proteins that remain soluble under these conditions are known as serum or whey proteins. Approximately 80% of the total nitrogen in bovine, ovine, caprine and buffalo milks is casein but casein represents only ~ 40% of the proteins in human milk. Both caseins and

whey proteins are quite heterogeneous and have very different molecular and physiochemical properties

The proteins of milk are, arguably, its most important constituents from the nutritional and physiological viewpoints. Many of these milk proteins also have distinctive physio-chemical, functional and technological properties, which are widely exploited in the food industry. The most important of these are:

- A remarkably high heat stability, which permits the manufacture of a range of heat-sterilized, concentrated and dehydrated products without major changes in the physical and organoleptic properties of milk;
- Coagulability with Ca^{2+} following limited rennet-induced proteolysis, which is exploited in the manufacture of a wide range of cheeses and some functional proteins;
- Coagulability at their isoelectric point (~ pH 4.6), which is exploited in the production of fermented dairy products, fresh cheeses and most functional milk proteins (Fox, 2001).

2.9.1 Classes of milk proteins

Milk contains hundreds of types of protein, most of them in very small amounts. The proteins can be classified in various ways according to their chemical or physical properties and their biological functions. The old way of grouping milk proteins into casein, albumin and globulin has given way to a more adequate classification system. Table 2.12 shows an abridged list of milk proteins according to a modern system (Teknotext AB, 1995).

Table 2.12 Concentration of proteins in milk.

	Conc. in milk g/kg	% of total protein w/w
Casein		
α_{s1}-casein	10.0	30.6
α_{s2}-casein	2.6	8.0
β-casein	10.1	30.8
κ-casein	3.3	10.1
Total casein	26.0	79.5
Whey proteins		
α-lactalbumin	1.2	3.7
β-lactoglobulin	3.2	9.8
Blood serum albumin	0.4	1.2
Immunoglobulins	0.7	2.1
Miscellaneous (including proteose-peptone)	0.8	2.4
Total whey proteins	0.8	2.4
Fat globule membrane proteins	0.4	1.2
Total protein	32.7	100

Source: Jelen, 1985.

2.9.2 Caseins

The casein content of milk represents about 80% of milk proteins. The principal casein fractions are α_{s1} and α_{s2}-caseins, ß -casein, and κ-casein. The distinguishing property of all caseins is their low solubility at pH 4.6. The common compositional factor is that caseins are conjugated proteins, most with phosphate group(s) esterified to serine residues. These phosphate groups are important to the structure of the casein micelle. Calcium binding by the individual caseins is proportional to the phosphate content.

The conformation of caseins is much like that of denatured globular proteins. The high number of proline residues in caseins causes particular bending of the protein chain and inhibits the formation of close-packed, ordered secondary structures. Caseins contain no disulfide bonds. As well, the lack of tertiary structure accounts for the stability of caseins against heat denaturation because there is very little structure to unfold. Without a tertiary structure there is considerable exposure of hydrophobic residues. This results in strong association reactions of the caseins and renders them insoluble in water.

Within the group of caseins, there are several distinguishing features based on their charge distribution and sensitivity to calcium precipitation as outlined in Table 2.13.

Table 2.13 Molecular structure and properties of the major milk proteins

Food protein	Protein component	% of total protein	Molecular weight	Isoelectric point	Molecular structure
Caseins	αs_1-Caseins	32-40	22,068-23,724	4.40-4.76	Primarily random coiled. Rich in pro and hydrophobic amino acids. Strong tendency for association by hydrophobic interactions.
	αs_2-Caseins	8-10	25,230	4.20-4.60	Primarily random coiled. Most hydrophilic of major caseins.
	β-Caseins	24-29	23,944-24,092	4.83-5.07	Either highly asymmetrical or random coiled. Most hydrophobic of major caseins. Strong tendency for association by hydrophobic interactions.
	κ-Caseins	5-10	19,007-19,039	5.30-5.49	Random coiled, containing distinct hydrophobic (para-κ-casein) and hydrophilic (macropeptide) regions. Tendency for association by disulfide bonds and hydrophobic interactions.
Whey proteins	β-Lactoglobulin	8-17	18,205-18,363	5.35-5.49	Globular protein, containing one cysteine and two cystine residues. Formation of dimers in pH range 5.2-7.5; octamerization may occur at pH 3.5-5.2.
	α-Lactalbumin	2-7	14,147-14,175	4.2-4.5	Compact globular protein, containing four residues. High stability between pH 5.4 and pH 9.0.
	Bovine serum albumin	2	66,267	4.7-4.9	Rod-shaped protein, containing one cysteine and 17 cystine residues. Partial unfolding at low (<4) and high (>8) pH values.
	Immunoglobulins	2-4	150,000-1,000,000	5.5-8.3	Monomers of polymers of four-chain molecular containing two light (MW 20,000) and two heavy (MW 50,000-70,000) chains linked by disulfide bonds.

2.9.1.2 *Structure: The Casein Micelle*

Most, but not all, of the casein proteins exist in a colloidal particle known as the casein micelle. Its biological function is to carry large amounts of highly insoluble CaP to mammalian young in liquid form and to form a clot in the stomach for more efficient nutrition. Besides casein protein, calcium and phosphate, the micelle also contains citrate, minor ions, lipase and plasmin enzymes, and entrapped milk serum. These micelles are rather porous structures, occupying about 4 ml/g and 6-12% of the total volume fraction of milk.

The "casein sub-micelle" model has been prominent for the last several years (Figure 2.9) but there is no universal acceptance of this model, and mounting research evidence to suggest that there is not a defined sub-micellar structure to the micelle at all. In the submicelle model, it is thought that there are small aggregates of whole casein, containing 10 to 100 casein molecules, called submicelles (Figure 2.8). It is thought that there are two different kinds of submicelle; with and without κ-casein. These submicelles contain a hydrophobic core and are covered by a hydrophilic coat which is at least partly comprised of the polar moieties of κ-casein. The hydrophilic caseinomacropeptide (CMP) of the κ-casein exists as a flexible hair.

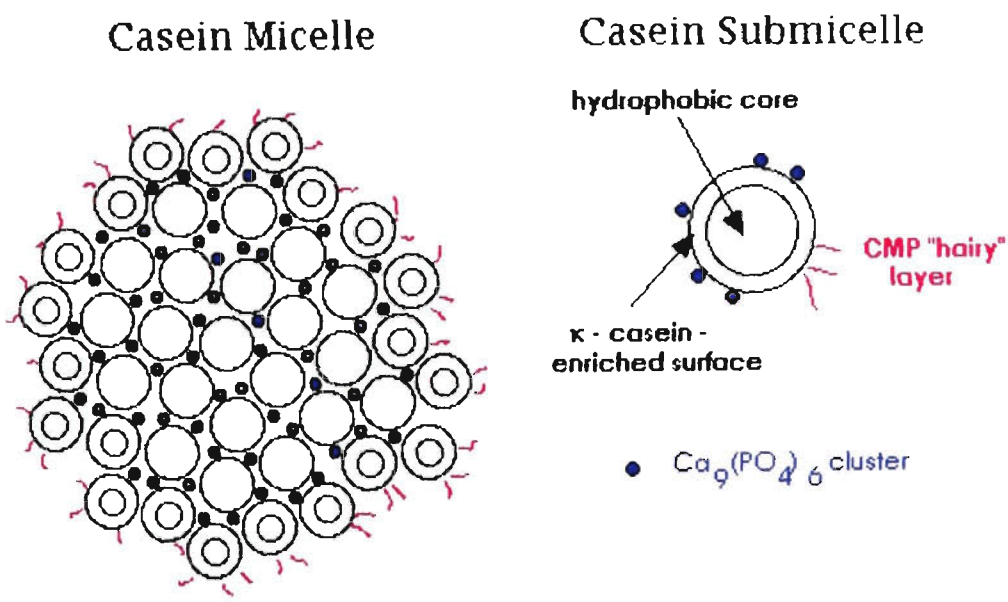

Figure 2.8 Casein micelle and sub-micelle model (Anon, 2003)

The open model (Figure 2.9) also suggests there are denser and less dense regions within the micelle, but there is less of a well-defined structure. In this model, calcium phosphate nanoclusters bind caseins and provide for the differences in density within the casein micelle.

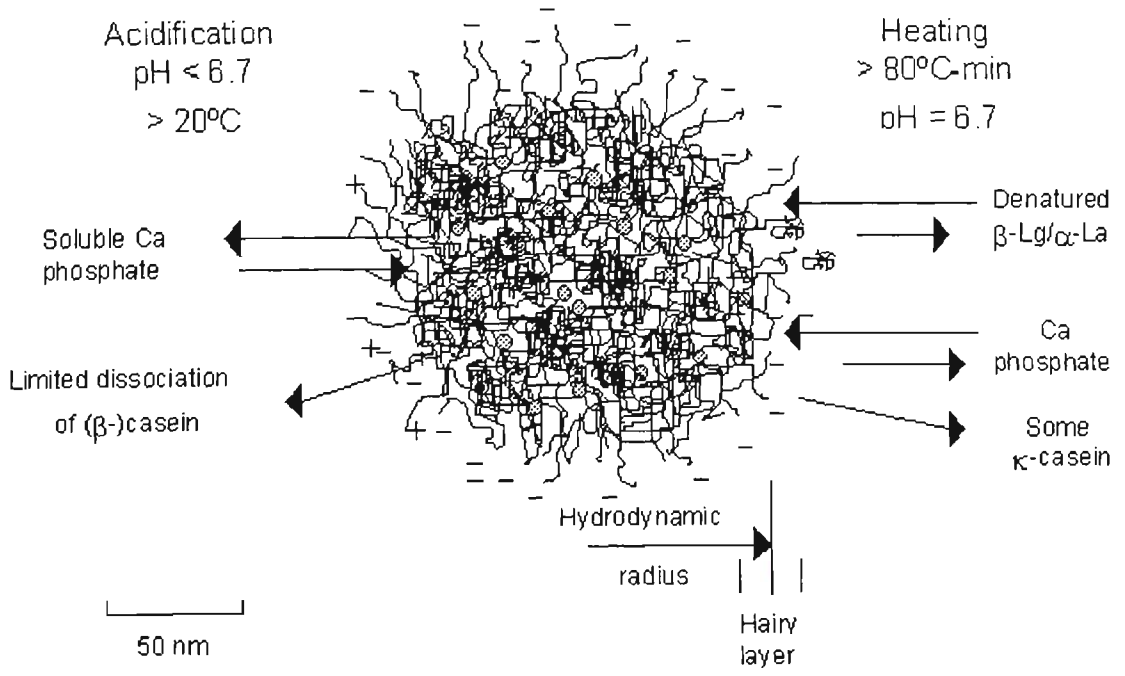

Figure 2.9 Casein submicelle open model (Anon, 2003).

2.9.3 Whey proteins

The whey protein fraction of bovine milk contains four main proteins: β-lactoglobulin (β-Lg, 50%), α-lactalbumin (α-La, 20%), blood serum albumin (BSA, 10%) and immunoglobulins (Ig, 10%; mainly IgG_1, with lesser amounts of IgG_2, IgA and IgM). Human milk contains no β-Lg and the principal Ig is IgA. The principal whey proteins are well characterized (Table 2.13). In contrast to the caseins, the whey proteins possess high levels of secondary, tertiary and, in most cases, quaternary structures. They are typical globular proteins and are denatured on heating, e.g. completely at 90°C × 10 min. They are not phosphorylated and are insensitive to Ca^{2+}. All whey proteins contain intramolecular disulphide bonds that stabilize their structure. β-Lg contains one sulphydryl group that is buried within the molecule in the native protein but becomes

exposed and active on denaturation of the protein by various agents (including heat) and can then undergo sulphydryl-disulphide interactions with itself or other proteins, the most important of which being that with κ-casein, which occurs on heating to > ~ 75°C × 15 s. This interaction markedly affects many technologically important properties, the structure, rheological and synertic properties of rennet- and acid-coagulated milk gels (including yoghurt, fresh and rennet-coagulated cheeses) and the heat stability of milk and concentrated milk products (Fox, 2001).

The whey proteins are known to exhibit biological activity, such as carrying of calcium, zinc, copper, iron and phosphate ions in the body (Table 2.14). Also, the caseins act as precursors of a number of different bioactive peptides (Korhonen *et al.*, 1998).

Table 2.14 Biological activity of milk proteins.

Protein	Concentration g/L	Function
Caseins (α, β and κ)	28	Ion carrier (Ca, PO_4, Fe, Zn, Cu) precursors of bioactive peptides
β-lactoglobulin	1.3	Retinol carrier, fatty acids binding, possible antioxidant
α-lactalbumin	1.2	Lactose synthesis in mammary gland, Ca carrier, immunomodulation, anticarcinogenic
Immunoglobulins A, M and G	0.7	Immune protection
Glycomacropeptide	1.2	Antiviral, bifidogenic
Lactoferrin (LF)	0.1	Antimicrobial, antioxidative, immunomodulation, iron absorption, anticarcinogenic
Lactoperoxidase	0.03	Antimicrobial
Lysozyme	0.0004	Antimicrobial, synergistic effect with immunoglobulins and LF

Source from Korhonen *et al.* (1998).

Many amongst milk proteins exhibit biological activities such as iron (Lf), fatty acid binding (β-Lg) properties as well as protective (Ig, lysozyme) effects in newborn. Otherwise, milk proteins are known to be precursors of biologically active peptides. Milk proteins are usually separated into classes before chromatographic analysis. Acid precipitation at pH 4.6 or rennet coagulation allows keeping whey proteins in solution. However, other methods such as ultra-centrifugation, gel filtration as well as membrane

technologies (ultrafiltration, microfiltration) on a large scale can be used to separate whole caseins from whey proteins (Léonil *et al.*, 2000).

2.10 Proteolytic system of lactic acid bacteria

2.10.1 Breakdown of protein

The process where protein is broken down is called proteolysis where "proteo" stands for protein and "lysis" for breakdown. The major enzymes concerned are proteases, e.g. rennin, pepsin and trypsin. These enzymes degrade proteins into peptides, which are then degradable by various peptidases to smaller peptides and free amino acids. Amino acids can be reutilized for protein synthesis by the cell; however, they can also be broken down oxidatively or fermentatively (Teknotext AB, 1995).

Proteins and their constituent amino acids have a wide combination of chemical elements and contain carbon, hydrogen, oxygen, sulphur, nitrogen and phosphorous. Breakdown of protein therefore results in a much larger range of acids, alcohols, gases (hydrogen, carbon dioxide, hydrogen sulphide and ammonia) and other compounds. Breakdown of protein nearly always results in ammonia, which is alkaline and has a strong smell (Teknotext AB, 1995).

Breakdown of protein in liquid milk takes place in two major stages called peptonisation and consists of curdling (sweet as opposed to sour) or clotting of the milk by rennin-like enzymes and proteolysis of the protein, resulting in production of ammonia, which is alkaline (Teknotext AB, 1995).

2.10.2 Protein metabolism

Although yoghurt starter cultures are considered to be only weakly proteolytic, *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* may, during the fermentation, cause a significant degree of proteolysis, and this activity may be important for the following reasons: the enzymatic hydrolysis of milk proteins results in the liberation of peptides of varying sizes and free amino acids, and these possible changes can affect the physical

structure of yoghurt; as discussed elsewhere, the liberation of amino acids into the milk is essential to the growth of *S. thermophilus* and although amino acids and peptides may not contribute directly towards the flavour of yoghurt, they do act as precursors for the multitude of reactions which produce flavour compounds. The range of products released by proteolysis is dependent on two main factors: firstly, the components of the milk protein fraction, and secondly, the types of proteolytic enzymes that the yoghurt organisms may possess (Tamime & Robinson, 1985).

2.10.3 Proteolytic enzymes

These enzymes, as the name suggests, are specific in their action, and their main function is to catalyse the hydrolytic cleavage of the peptide bonds which form the backbone of the protein molecule. Enzymes acting on peptide bonds are known as peptide hydrolases, and up to the present time a large number of such enzymes have been identified. In the past the name given to an enzyme was derived from the substrate involved, but this approach has created such confusion in the field of enzymology, that the Nomenclature Committee of the International Union of Biochemistry was established to consider a classification of universal application to enzymes and coenzymes. The peptide hydrolases are divided into two main groups, i.e. the peptidases and the proteinases (Tamime & Robinson, 1985).

The hydrolysis of protein to yield amino acids can, therefore, be accomplished in two major stages:

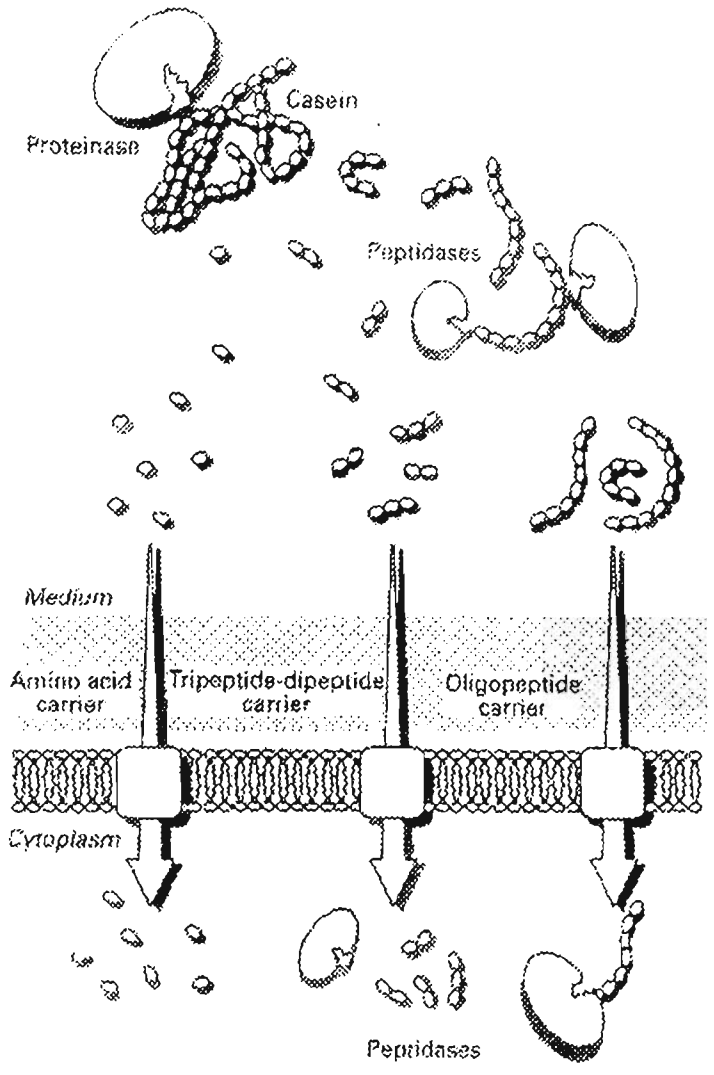

Figure 2.10 Proposed scheme for the breakdown and utilization of casein by lactococci (Tan *et al.*, 1993).

2.10.3.1 Peptide hydrolases of the yoghurt organisms

The data compiled by Tamime and Deeth (1980) on the proteolytic activity of *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus;* indicate that both organisms possess different peptidases and proteinases. The former organism is considered to have more peptidase activity than *L. delbrueckii* ssp. *bulgaricus*, and only limited proteinase activity, while the ability of *L. delbrueckii* ssp. *bulgaricus* to hydrolyse casein confirms that proteinase activity is much higher in the lactobacilli. This pattern of peptide hydrolysis in

the yoghurt organisms provides further evidence of the symbiotic relationship which exists between *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus*. Thus the proteinase activity of *L. delbrueckii* ssp. *bulgaricus* hydrolyses the casein to yield polypeptides, which in turn are broken down by the peptidases of *S. thermophilus* with the liberation of amino acids (Tamime & Robinson, 1985).

Several proteolytic enzymes have been purified and biochemically characterized, and in some cases the corresponding genes have been analysed. In addition, the properties of the transport systems of several amino acids and peptides have been studied in lactococci (Tan *et al.*, 1993). Several peptidases of LAB have been isolated and characterized. Endopeptidases hydrolyze large casein fragments into smaller peptides which can be degraded by several aminopeptidases. Specific X-prolyl-dipeptidylamino-peptidases degrade the proline-rich peptides while di- and tri-peptidases and prolidases release free amino acids, which finally complete the degradation of casein (Tan *et al.*, 1991). Several peptidases have been purified from *Lactococcus lactis*: two endopeptidases, a tripeptidase, some aminopeptidases and X-prolyl-dipeptidyl aminopeptidases, a prolidases, and two dipeptidases. Only a few peptidases have been isolated from Streptococcus, Lactobacillus and bifidobacteria species to date as summarized in Table 2.15.

Table 2.15 Peptidases purified from yoghurt and probiotic bacteria

Peptidase	Organism	Substrate	Mwt kDa	Reference
General aminopeptidase	*L. acidophilus* R-26	Lys-pNA[1]	38	Machuga & Ives (1984)
	L. delbrueckii ssp. *bulgaricus* CNRZ397	Lys-pNA	95	Atlan *et al.* (1989) Bockelmann *et al.* (1992)
	L. delbrueckii ssp. *bulgaricus* B14	Lys-pNA	95	Tsakalidou & Kalantzopoulos (1992)
	S. thermophilus	Lys-pNA	89	Cheng & Nagasawa (1985)
	Bifidobacterium breve	Lys-pNA		
X-prolyl-dipeptidyl aminopeptidases	*L. delbrueckii* CNRZ397	Gly-Pro-pNA[2]	82	Atlan *et al.* (1990)
	L. delbrueckii ssp. *bulgaricus* B14	Gly-Pro-pNA	95	Bockelmann *et al.* (1991)
	L. delbrueckii LBU147		90	Miyakawa *et al.* (1991)
	L. acidophilus		95	Bockelmann *et al.* (1991)
	S. thermophilus			Meyer & Jordi (1987)
Proline iminopeptidase	*L. delbrueckii* ssp. *bulgaricus* CNRZ397			Atlan *et al.* (1990)
Dipeptidase	*L. delbrueckii* ssp. *bulgaricus* B14	Leu-Leu[3]	51	Wohlrab & Bockelmann (1992)
	-*S. thermophilus*		50	Rabier & Desmazeaud (1973)
Tripeptidases	*L. delbrueckii* ssp. *bulgaricus* B14			Bockelmann *et al.* (1995)
Endopeptidases	*L. delbrueckii* ssp. *bulgaricus*		70	Bockelmann *et al.* (1996)

[1] Lys-pNa = lysyl-p-nitroanilide; [2] Gly-Pro-pNA = glycyl-prolyl-p-nitroanilide; [3] Leu-Leu = leucyl-leucine

Assays of *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* for peptide hydrolases reveal that such enzymes are either cell bound and/or are intracellular. However, Miller and Kandler (1967a) reported that *L. delbrueckii* ssp. *bulgaricus* possess an extracellular protease, an observation which was not confirmed by other workers. Based on the data compiled by Tamime and Deeth (1985), the activity of the peptide hydrolases of the yoghurt organisms appears to be at maximum under the following conditions:

- Most intense activity is during the log phase.
- The rate of proteolysis decreases during storage, or after the stationary phase has been reached.

The ratio of *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* in the starter culture can affect the level of amino acids in yoghurt, and for example, 70 µg/100mL is liberated at a ratio of 1:1, followed by 50 µg/100mL at 1:2 and 41 µg/100mL at 2:1. However, the acidity of these yoghurts was rather high, i.e. 1.9% lactic acid for the 1:1 ratio, and it is possible that the high level of liberated amino acids in the product was associated with the proteolytic activity of *L. delbrueckii* ssp. *bulgaricus* which becomes the predominant organism in such an acidic environment (Tamime & Robinson, 1985).

In yoghurt (24 hours old) the spectrum of amino acids changes in relation to the ratio of cocci:rods, i.e. at a ratio of 1:1, tyrosine, phenylalanine and leucine formed 56% of the amino acid pool, but at a ratio of 3:1 proline accounted for 71% of the free amino acids (Tamime & Robinson, 1985).

- The hydrolysis of whey proteins in milk yields lower levels of non-protein nitrogen as the ratio of *L. delbrueckii* ssp. *bulgaricus* to *S. thermophilus* is decreased.
- Free fatty acids, e.g. capric and, to a lesser degree, oleic, can reduce the proteolytic activity of the starter cultures, and can affect the texture of the coagulum.
- Enhanced proteolytic activity in yoghurt is observed during the manufacture of lactose hydrolysed yoghurt, due perhaps, to protease residues present in the β-D-galactosidase preparations.
- Milk precultured with psychotrophic bacteria prior to the manufacture of yoghurt was found to support enhanced proteolytic activity; however, the product developed unacceptable flavours.

Bitterness in yoghurt is usually attributed to the production of bitter peptides by the proteolytic activity of *L. delbrueckii* ssp. *bulgaricus*; however, fermentation of the milk at 44°C yields yoghurt which is less likely to be bitter than yoghurt produced at 38°C (Tamime & Robinson, 1985).

2.10.3.2 Products of proteolysis

The profile of nitrogenous compounds in yoghurt, as compared with milk changes due to the proteolytic activity of *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus*, both during the fermentation period and, to a lesser degree, during the cold storage of the product. Basically, the change amounts to an increase in the level of soluble nitrogenous compounds, which also includes the liberation of amino acids, and the release of peptides from the milk proteins (Tamime & Robinson, 1985).

The most comprehensive study in this field was conducted by Miller and Kandler (1967a, b) where they confirmed that different strains of yoghurt organisms vary in their proteolytic activity, and further, that the amounts of dialyzable nitrogen released by *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus* (490 and 302 mg/litre) are compliant with the view that the former organism is more proteolytic than *S. thermophilus*. The same trend can be observed in relation to the amounts of amino acid nitrogen, urea nitrogen and peptide nitrogen, but the special capacity of *S. thermophilus* to increase the level of ammonia nitrogen in cultured milks is due to the ability of the lactic streptococci to split urea (Tamime & Robinson, 1985).

The spectrum of free amino acids in milk and yoghurt (see Table 2.16) is dependent on several variables such as follows:

(a) *Type of milk.* Milks from different species (cow, sheep or goat) have different contents of amino acids, i.e. $\leq$ 10, 3.78 and 20.6 mg/100 ml respectively, and in addition, goat's milk has, relative to the others, much higher levels of alanine, glycine, glutamic acid, serine, and threonine.

(b) *Methods of manufacture.* Slightly higher levels of amino acids are obtained when the fermentation is carried out at 42°C for 2-3 hours, rather that at 42°C for 1 hour followed by 5-6 hours at 30-32°; the total amino acid contents of such yoghurts were 23.6 and 19.4 mg/100 ml.

(c) *Ratio of rods to cocci.* Due to the fact that *L. delbrueckii* ssp. *bulgaricus* is more proteolytic than *S. thermophilus*, the higher the ratio of rods to cocci in the starter

culture, the higher the amino acid content is likely to be in the corresponding yoghurt.

(d) *Conditions during storage.* The temperature of storage of yoghurt can affect the level of free amino acids in the product, i.e. the higher the storage temperature, the greater the increase in free amino acids. It has been reported that storing full and low (1%) fat natural yoghurts at 4°C and 20°C for a duration of 60 days, produced an increase in the level of amino acids in these yoghurts (at 4°C) 2.36 and 1.00, and (at 20°C) 7.57 and 14.56 mg/100 ml respectively. However, other studies by the same workers observed no increase in the level of amino acids in lemon and orange flavoured yoghurts stored under the same conditions for the same period of time, a difference that was attributed to the presence of natural metabolic inhibitors in the fruit, or the effect of some bacteriocidal agent added to the fruit concentrate, or the high acidity of the fruit preparation.

(e) *Level of lactic acid.* The amino acid content of yoghurt is dependent on the titratable acidity of the product. Studies have shown that yoghurts which contained 1.9 and 1.72-1.73% lactic acid had total amino acid contents of 70 and 41-50 mg/100 g respectively.

The final amino acid content of yoghurt made from cow's milk may range from 18.7-33 mg/100 ml (see Table 2.16), and it is probable that the acidities of these yoghurts were 1.0-1.4% lactic acid. It is important, of course, that the total amino acid content of yoghurt reflects a balance between proteolysis and assimilation by the bacteria. Some amino acids, such as glutamic acid, proline and, to a lesser degree, alanine and serine, are presumably not required by the yoghurt organisms, and thus accumulate in larger quantities in the product than the remaining amino acids which are utilized by *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* during growth and/or fermentation (Tamime & Robinson, 1985).

Table 2.16 Free amino acid content (mg/100 ml) of milk and yoghurt

	Cow's		Goat's		Sheep's	
Amino acid	**Milk**	**Yoghurt**	**Milk**	**Yoghurt**	**Milk**	**Yoghurt**
Alanine	0.16-0.64	1.17-3.80	1.33	3.83	0.56	1.30
Arginine	0.16-0.96	0.70-1.39	0.40	0.67	0.26	0.85
Aspartic acid	0.23-0.52	0.70-1.20	0.22	1.37	0.18	1.75
Glycine	0.30-0.53	0.28-0.45	5.91	6.06	0.15	0.25
Glutamic acid	1.48-3.90	4.80-7.06	3.54	3.78	1.08	4.10
Histidine	0.11	0.80-1.70	0.45	1.28	0.10	0.50
Isoleucine	0.06-0.15	0.15-0.40	0.18	0.43	0.06	0.25
Leucine	0.06-0.26	0.70-1.82	0.21	1.25	0.23	0.45
Lysine	0.22-0.94	0.80-1.11	0.60	2.35	0.19	0.72
Methionine	0.05	0.08-0.20	0.10	0.35	0.05	0.15
Phenylalanine	0.05-0.13	0.17-0.61	0.11	0.35	0.08	0.15
Proline	0.12	5.40-7.05	0.65	4.35	0.11	4.30
Serine	0.08-1.35	1.50-2.90	3.05	3.51	0.20	2.00
Threonine	0.05-0.26	0.24-0.70	3.34	2.80	0.13	0.55
Tryptophan	Trace	0.2	No reported value		No reported value	
Tyrosine	0.06-0.14	0.18-0.61	0.30	0.60	0.16	0.24
Valine	0.10-0.25	0.90-1.86	0.30	0.50	0.24	0.90
Total	3.29-10.31	18.77-33.06	20.60	33.48	3.78	18.46

Source: Tamime & Robinson (1985).

2.10.4 Methods for quantification of milk proteins

Quantification has been done separately for casein and whey proteins by using independent runs of gel electrophoresis, liquid chromatography (Visser *et al.*, 1991; Léonil *et al.*, 1995), and capillary electrophoresis (de Jong *et al.*, 1993; Otte *et al.*, 1994) Simultaneous separation and quantification of the casein and whey proteins has been reported by capillary zone electrophoresis, isoelectric focusing, and high-performance liquid chromatography (HPLC). The method using capillary zone electrophoresis did not report α_{s2}-CN quantification (de Jong *et al.*, 1993). Moreover, methods using HPLC and isoelectric focusing did not separate α-LA and β-CN B (Visser *et al.*, 1991), α-LA and β-LG (Guillou *et al.*, 1987), or κ-CN B and αs2-CN (Léonil *et al.*, 1995).

2.10.4.1 Capillary electrophoresis

Traditionally, milk proteins have been separated by gel electrophoresis. Recently, ion exchange and reversed phase HPLC have also been employed. Capillary electrophoresis (CE) offers advantages in terms of speed and resolution for the analysis of proteins in complex samples. These analytical methods can provide relevant information for the development and manufacturing of dairy products. In addition, this method is a useful tool in controlling potential adulteration of milk (Tienstra *et al.*, 1993)

CE is a fast growing technique that is finding new applications in the analysis of milk proteins and peptides owing to its well known advantages over the electrophoretic and chromatographic methods. With no more than 20 years since its introduction of the first commercial instruments, the technique cannot be considered mature, relative to HPLC for example. In spite of this, CE has attracted great interest because it has many advantages compared to polyacrylamide gel electrophoresis (PAGE) (Otte *et a*l., 1994).

CE is performed in a narrow fused-silica capillary (20-200μm inner diameter) with an outer coating of polyimide to protect the glass fibre during handling. A short section of the polyimide coating is removed to form a window for direct detection through the capillary wall. Analytes are continuously monitored by an on-capillary detector, usually UV/VIS. This makes the technique fully automatable with the possibility of running several samples in sequence. UV absorbency also allows the detection of small molecules like peptides and amino acids which are impossible to visualize in PAGE because of problems with fixing and staining. Furthermore, UV detection enables quantitative analysis in CE with an accuracy exceeding that is possible in PAGE (Otte *et al.*, 1994).

CE resolves molecules, based on charge/mass ratio, in a buffer-filled fused-silica capillary under the influence of an electric field. The use of high electrical fields results in short analysis times and high efficiency and resolution. Like classical gel electrophoretic techniques, CE has diversified. The basic methods of CE include capillary zone electrophoresis (CZE), often referred to as free-solution, CE (FSCE),

micellar electrokinetic chromatography (MECC), capillary gel electrophoresis (CGE), capillary isoelectric focusing (CIEF) and capillary isotachophoresis (CITP) (Otte *et al.* 1994).

2.10.4.2 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Alternate methods for quantitation of caseins and whey proteins in milk products have been investigated. The Harland-Ashworth and Leighton procedures, which are used for routine determination of soluble whey proteins in milk, could not be adapted satisfactorily to quantitation of whey protein in blends of nonfat dry milk solids and whey protein concentrates because of problems of precipitation techniques. Gel electrophoresis in sodium dodecyl sulfate does not require fractionation prior to analysis and works well for whey protein concentrates, as well as total milk protein concentrates (Basch *et al.*, 1985).

SDS-PAGE is the most widely used method for qualitatively analyzing protein mixtures. It is particularly useful for monitoring protein purification, and because the method is based on the separation of proteins according to size, the method can also be used to determine the relative molecular mass of proteins. The most widely used system for SDS-PAGE is the method described by Laemmli (Walker, 1996).

2.10.4.3 Hydrolysis of casein

The importance of breakdown of casein into water soluble compounds with the action of proteolytic enzymes by microorganisms is now well recognized. LAB are known to be caseinolytic, although the extent of degradation varies with different strains (Singh & Sharma, 1983).

Proteolysis of whole bovine casein has been used to study the total proteolytic activity of lactic acid bacteria strains (Kalantzopoulos *et al.*, 1990; Abrahim *et al.*, 1993). Previous studies have shown that from electrophoretic patterns obtained after incubation with

casein, it was observed that there was a higher initial proteolytic activity occurring in lactobacilli than in streptococci (Kalantzopoulos *et al.*, 1990).

2.10.4.4 *High performance liquid chromatography of milk proteins*

The qualitative and quantitative analysis of proteins or protein digests by different high-performance liquid chromatography (HPLC) modes has become routine in most laboratories dealing with protein research and food analysis. There is no doubt that the present high-resolution chromatographic techniques offer many advantages for the separation and analysis of proteins. However, it has been recognized that the chromatography of proteins is in many respects different from that of small, more "rigid" biomolecules such as saccharides and organic acids. Modern laboratory practice requires not only a complete resolution of all compounds in a mixture in a short time but also, especially on a preparative scale, the highest possible recovery of sensitivity and resolution of modern HPLC systems.

Unless whole milk is to be analyzed, sample preparation usually includes the separation of the casein fraction from the whey fraction. This can be achieved during processing of the food, e.g., during the production of caseinates, whey protein isolates (WPIs), or whey protein concentrates (WPCs), or it has to be done prior to analysis. Possible means are centrifugation of the skim milk in the presence of Ca2+ to precipitate the casein fraction, renneting of the milk to obtain sweet whey, acidification of the milk to pH 4.6, with acid or lactic acid bacteria, to obtain acid whey. Acidification to pH 4.6 can also be used to remove denatured whey proteins. Other sample pretreatments can include the removal of low-molecular-weight compounds, such as salts and lactose, by dialysis, ultracentrifugation, and the addition of salts, e.g., $(NH_4)_2SO_4$, to remove aggregated, denatured proteins.

Chapter 3 Materials and Methods

3.1 Materials

3.1.1 General Chemicals and Materials

D-sorbitol, lithium chloride, nalidixic acid, neomycin sulfate, paromomycin sulfate, L-cysteine hydrochloride (monohydrate), o-phthaldialdehyde (OPA), trichloroacetic acid (TCA), sodium dodecyl sulfate (SDS), β-mercaptoethanol, β-lactoglobulin, β-lactoglobulin A, β-lactoglobulin B, α-lactalbumin, α-casein, β-casein, κ-casein, acrylamide, N, Ń-methylenebis acrylamide, ammonium persulfate, casein, N, N, Ń, Ń-tetramethylethylenediamine (TEMED), coomassie brilliant blue (R250), Folin-ciocalteu, salicin, L-leucine-ρ-nitroanilide, L-lysine-ρ-nitroanilide, L-alanine-ρ-nitroanilide, L-proline-ρ-nitroanilide, L-arginine-ρ-nitroanilide, L-methionine-ρ-nitroanilide, Tris-HCl, Ala-Met, Leu-Tyr, Leu-Gly, Ala-His, Pro-Ile, peroxidase, L-amino acid oxidase, o-dianisidine, dithiothreitol, Gly-Ala-Tyr, Gly-Leu-Phe, Gly-Gly-Phe, bradykinin, Ala-Ala-Ala-Ala-Ala, magnesium chloride, DNase, RNase, proteinase K, methylhydroxy-propylcellulose (MHPC), 5-sulfosalicylic acid, cysteine, methionine, threonine, aspartic acid, arginine, bromocresol purple, glycerol, bromophenol blue, brilliant blue R, chymotrypsin and papain were all purchased from Sigma-Aldrich Chemical Co. (St Louis, MO, USA).

AnalaR grade chemicals of D-glucose, sodium hydroxide pellets, sucrose, hydrochloric acid (36%), glacial acetic acid, absolute ethanol (99.7-100% v/v), anhydrous sodium acetate, formaldehyde, anhydrous sodium carbonate, ethylenediamine-tetra-acetic acid disodium salt (EDTA), urea, citric acid and alanine (BDH grade) were all purchased from Merck, Crown Scientific Pty. Ltd. (Rowville, Vic, Australia).

GPR grade chemicals of copper sulfate, potassium tartrate, sodium carbonate, anhydrous di-potassium hydrogen phosphate (K_2HPO_4), anhydrous sodium dihydrogen orthophosphate (Na_2HPO_4), and sodium chloride were all purchased from Merck, Crown Scientific Pty. Ltd. (Rowville, Vic, Australia). Methanol (HiPerSolv for

chromatography), anhydrous sodium tetraborate, anhydrous trisodium phosphate were supplied by Aldrich, Sigma-Aldrich. Silver nitrate was supplied by ICN, Biomedicals. Prestained SDS-PAGE broad range standards were purchased from Bio-Rad Laboratories (Hercules, CA USA). Coomassie® protein assay reagent and albumin standard was purchased from Pierce (Rockford, IL USA).

3.1.2 Media

Peptone water, tryptone, yeast extract, agar powder, dehydrated MRS broth, dehydrated MSR agar were all purchased from Oxoid (West Heidelberg, Australia). MRS broth without carbohydrates (Code AM 103-NC) and supplement (SP437) were purchased from Amyl media,

3.1.3 Starter cultures

Four commercial starter cultures (ABY-1, ABY-2, ABT-1 and ABT-4) were used in this research project. Cultures ABY-1 and ABY-2 contained *Streptococcus thermophilus*, *Lactobacillus delbrueckii* ssp. *bulgaricus*, *Lactobacillus acidophilus* and *bifidobacteria* as constitutive microflora, whereas cultures ABT-1 and ABT-4 contained *S. thermophilus*, *L. acidophilus* and bifidobacteria only. In all the commercial culture combinations studied, the strains of probiotic organisms (*L. acidophilus* and bifidobacteria) were kept the same, whereas strains of yoghurt bacteria varied. Culture ABT-1 contained a strain of *S. thermophilus* that produces polysaccharides during fermentation. The organisms were previously characterised and identified (Dave, 1998). The starter cultures were in freeze dried DVS (Direct Vat System) form and were obtained from Chr. Hansen Pty. Ltd. (Bayswater, Australia). After procurement, the starter cultures were stored at -18°C in absence of atmospheric air.

3.1.4 Sources of lactic acid bacteria

Various strains of lactic acid bacteria (LAB) used in this study and their sources are given in Table 3.1. The organisms were activated by three successive transfers in suitable media. All LAB were stored as liquid stock cultures in reconstituted skim milk (12% nonfat dry milk) supplemented with glucose (2% w/v) and yeast extract (1% w/v). The

stock cultures were stored at -20°C (short-term storage) and -80°C (long-term storage). After 20 transfers of the organisms, a fresh transfer of cultures was taken from the original frozen stock culture to avoid loss of plasmid associated properties.

Table 3.1 Sources of various lactic acid bacterial strains

Organism	Strain/s	Total No.	Source
Lactobacillus acidophilus (LA)	2400, 2401, 2404, 2405, 2406, 2409, 2410, 2411, 2412, 2413, 2414, 2415, 2422	13	Victoria University culture collection[1]
	T-1 LA (from ABT-1)	1	Chr. Hansen[2]
Lactobacillus delbrueckii ssp. *bulgaricus* (LB)	2501, 2505, 2515, 2517, 2519	5	Victoria University culture collection
	Y-2 (from ABY-2)	1	Chr. Hansen
Streptococcus thermophilus (ST)	2000, 2002, 2008, 2010, 2013, 2014	6	Victoria University culture collection
	T-1 (ABT-1), T4 (ABT-4), Y-1 (ABY-1)	3	Chr. Hansen
Bifidobacterium ssp. (BB)	5090, 5095, 1912, 1900, 1920, 20097, 5094, 20099, 5089, 20210, 1941, 5092	12	Victoria University culture collection
	B-2 (from ABY-2)	1	Chr. Hansen

[1] Victoria University, School of Molecular Sciences, Werribee, Australia.
[2] Isolated from AB starter cultures manufactures by Chr. Hansen Pty. Ltd. (Horshlom, Denmark).

3.1.5 Media requirements and preparation

3.1.5.1 Peptone and water diluent

Peptone and water diluent (1.5%) was prepared by dissolving 15g of peptone water medium (Oxoid, West Heidelberg, Australia) in 1000mL of distilled water, adjusting the pH to 7.0 ± 0.2, followed by autoclaving 9.2mL aliquots at 121°C for 15 minutes.

3.1.5.2 Streptococcus thermophilus agar

The ingredients of *Streptococcus thermophilus* (ST) agar (10.0g of tryptone; 10.0g of sucrose; 5g of yeast extract and 2.0g of K_2HPO_4) were dissolved in 1000mL of distilled water, the pH was adjusted to 6.8 ± 0.1, and 6mL of 0.5% bromocresol purple was added

to the medium. Agar powder was added at the rate of 1.2% to the medium, which was sterilized at 121°C for 15 minutes.

3.1.5.3 MRS agar and pH-Modified MRS agar (pH 5.2)

Dehydrated MRS broth (Oxoid, Australia) was prepared as per the instructions of the manufacturer. To obtain pH-modified MRS agars, 1.0M HCl was used to adjust the pH of the medium to 5.2. After the broth was prepared, agar powder was added at the rate of 1.0%, and the media was autoclaved at 121°C for 15 minutes.

3.1.5.4 MRS-sorbitol agar

To prepare MRS-sorbitol agar, MRS basal medium without dextrose was prepared, and 10mL of 10% membrane-filtered solutions of D-sorbitol (Sigma, Castle Hill, Australia) were added per 90mL of basal medium (final concentration, 1.0%) just before pouring the agar medium.

3.1.5.5 MRS-NNLP agar

The MRS-NNLP (all chemicals from Sigma, Castle Hill, Australia) agar was prepared according to the method described by Laroia and Martin (1991). The basal medium was MRS agar. Filter-sterilized NNLP was added to the autoclaved MRS base just before pouring. Filter-sterilized L-cysteine-HCl (0.05% final concentration) was also added at the same time to lower the oxidation reduction potential of the medium and to enhance the growth of anaerobic bifidobacteria.

3.1.6 Standard Stock Solutions

3.1.6.1 SDS-PAGE solutions

Acrylamide stock solution (30%)

Acrylamide (29.1g) and 0.9g of N, N'-methylenebis-acrylamide were dissolved in 60mL of milli-Q water and the temperature was slightly increased to a temperature of 50°C. The volume of this solution was made up to 100mL with milli-Q water and stored in an amber coloured bottle at 4°C for up to 2 weeks.

Running buffer 1.875M tris-HCl buffer, pH 8.8

Tris (hydroxymethyl) aminomethane (56.8g) was added to 150mL of milli-Q water. The pH was carefully adjusted to 8.8 with concentrated HCl. The volume of this solution was made up to 25mL with milli-Q water and stored at 4°C for up to 4 weeks.

Stacking gel buffer 1.25M Tris-HCl pH 6.8

Tris (hydroxymethyl) aminomethane (37.8g) was dissolved in 150mL of water and stirred. The pH was carefully adjusted to 6.8 with concentrated HCl. The volume of the solution was made up to 250mL with water and stored at 4°C for up to 4 weeks.

10% (w/v) Sodium dodecylsulfate (SDS)

SDS (10g) was dissolved in 85mL of milli-Q water. The volume of the solution was made up to 100mL with water and stored at room temperature (~ 22°C) for up to 4 months.

10% Ammonium persulfate (APS)

APS (0.1g) was dissolved in 1mL of water. The solution was made fresh before use.

N, N, N', N'-tetramethylene-diamine (TEMED)

Ready made solution of TEMED was used.

10 × Electrode buffer (Tris-glycine)

Glycine (144.2g), 30.3g of tris and 10g of SDS were dissolved in 800mL of water. The volume of the solution was made up to 1000mL with water. The pH of this solution should was approximately 8.3 without adjustment. The solution was stored at room temperature for up to 2 months.

Reducing sample buffer (2 ×)

Glycerol (5.8mL of 87% solution), 2.5mL of 2-mercaptoethanol, 2.5mL of 1.25M tris-HCl (pH 6.8), 1g of SDS and 5-10mg of bromophenol blue were dissolved in 35mL of

water until all SDS and bromophenol blue were dissolved. Once dissolved, the loading buffer was made up to 50mL with water. Loading buffer was then aliquoted and stored at -20°C.

Marker proteins for molecular weight determination

Marker proteins with known molecular weights (broad range) were run on all gels to identify casein fractions in the samples.

3.1.6.2 Coomassie blue stain solutions

0.1% Coomassie blue stain solution

Coomassie brilliant blue (R250) (0.50g/500mL) was dissolved in water, methanol and acetic acid (5:5:2; vol/vol/vol).

Destain solution

Methanol (10%) was mixed with 7% glacial acetic acid.

3.1.6.3 Coomassie® Protein Assay solutions

Coomassie® protein assay reagent

The dye solution consists of Coomassie® Brilliant Blue G-250, phosphoric acid, methanol, water and solubilizing agents.

Bovine serum albumin (BSA) standard

Fraction V bovine serum albumin in 0.9% NaCl with sodium azide was used. The protein stock concentration was 2mg/mL.

3.1.6.4 Lowry protein determination solutions

Solution A

Solution A was prepared by mixing 1 part of 5% (w/v) copper sulfate; 9 parts of 1% (w/v) potassium tartrate and 100 parts of 10% (w/v) sodium carbonate in 0.5M sodium hydroxide.

Solution B

Solution B was prepared by mixing 1 part of Folin-Ciocalteu's reagent and 10 parts of water.

Bovine serum albumin (BSA) standard

Fraction V bovine serum albumin in 0.9% NaCl with sodium azide is used. The protein stock concentration is 2mg/mL.

3.2 Methods

3.2.1 Maintenance and propagation of bacterial cultures

Isolated and characterised bacterial cultures were maintained at -20°C (short-term storage) and -80°C (long-term storage) in 12% RSM supplemented with 2% glucose and 1% yeast extract. L-cysteine hydrochloride (0.05%) was additionally incorporated for bifidobacteria. From these frozen stock cultures, working cultures were made. Sterile 10mL batches of 12% RSM were inoculated with 1% of each culture and incubated at 37°C for 18 hours for *S. thermophilus, L. acidophilus* and bifidobacteria and 42°C for 18 hours for *L. delbrueckii* ssp. *bulgaricus*. All cultures were activated by subculture into fresh RSM on a weekly basis for a maximum of twenty subcultures before a new working culture was made from frozen stock cultures. This was done to minimise loss of any plasmid associated inhibitory activities.

3.2.2 Protocols used for the isolation, selective and differential enumeration of LAB

One gram of bacterial culture was ten fold serially diluted (10^3 to 10^7) with 9mL of 1.5% peptone and diluent water (Oxoid, W. Heidelberg, Australia) and mixed uniformly. Enumeration was carried out using the pour plate technique. The counts of *S. thermophilus* were enumerated on ST agar by incubating the plates aerobically at 37°C for 24 hours. MRS agar (Oxoid, Australia) adjusted to pH 5.2 and anaerobic incubation at 43°C for 72 hours were used for the differential enumeration of *L. delbrueckii* ssp. *bulgaricus*. *L. acidophilus* and *Bifidobacterium* ssp. were differentially enumerated using

MRS-sorbitol and MRS-NNLP (nalidixic acid, neomycin sulfate, lithium chloride and paromomycin sulfate) agar, respectively, and incubated anaerobically at 37°C for 72 hours (Dave & Shah, 1996). Oxoid jars and anaerobic kits were used to provide anaerobic conditions (Oxoid, Australia). Duplicate plates containing 25 to 250 colonies were enumerated and recorded as log of colony-forming units per gram of culture.

3.2.3 Proteolytic activity in reconstituted skim milk

3.2.3.1 Sample preparations

All the bacterial strains were grown overnight at 37°C in de Mann Rogosa Sharpe (MRS) broth (Oxoid, W. Heidelberg, Australia). To minimise carryover of free amino acids during inoculation, 5mL of cells were washed and resuspended to the original volume with 0.32mM sodium phosphate, pH 7.2. Cells were inoculated (1%) into RSM 12% (w/v) and incubated at their optimum temperatures (37°C for *S. thermophilus*, *L. acidophilus*, and *Bifidobacterium* ssp. and 42°C for *L. delbrueckii* ssp. *bulgaricus*) for 6 hours. A control consisted of uninoculated RSM. A 2.5mL sample of each incubated RSM was mixed with 10mL of 0.75M trichloroacetic acid (TCA) and 1mL of water to 5mL of sample to give a final concentration of 0.47M (7.7%) TCA. The samples were filtered using a Whatman number 4A filter paper after 10 minutes of incubation at room temperature (~22°C) and frozen at 80°C until assayed.

3.2.3.2 o-phthaldialdehyde procedure

The *o*-phthaldialdehyde (OPA) method described by Church *et al.* (1983) was used to determine the concentration of free amino groups in the filtrate. The OPA reagent was made by combining 25mL of 100mM sodium tetraborate, 2.5mL 20% (wt/wt) SDS, 40mg OPA dissolved in 1mL methanol, and 100μL β-mercaptoethanol, then adjusting the volume to 50mL with water. Triplicate aliquots from each TCA filtrate (1mL) were mixed with 1mL of OPA reagent in the cuvette and held for 2 minutes before reading absorbance at 340nm using a Pharmacia LKB Novaspek II Spectrophotometer (Pharmacia, Biotech, Uppsala, Sweden).

3.2.4 Enzyme assays

3.2.4.1 Preparation of intracellular and cell wall extracts

The bacteria were grown three times successively in 12% RSM to activate the organisms and finally in MRS broth (Oxoid, Australia), for the lactobacilli, and ST broth for the *S. thermophilus* to avoid carryover of milk proteins. The method of El Soda and Desmazeaud (1982) was used to prepare the intracellular and cell wall extracts. The growth was assessed by measuring the absorbance at 600nm. The cells in the late logarithmic phase (lactobacilli, 10 hours at 42°C; streptococci, 6 hours at 37°C and bifidobacteria, 16 hours at 37°C) were collected from the growth medium by centrifugation at 12,000*g* for 15 minutes at 4°C. The supernatant was designated the cell wall extracellular extract (CWE). The pellet obtained was washed twice with 0.9% (w/v) NaCl solution. The pellet was resuspended in 0.05M Tris-HCl buffer, pH 8.5 at 10% of volume of original growth medium and sonicated for 30 second intervals for 5 minutes at 43°C. The supernatant obtained after centrifugation (12,000*g* for 15 minutes at 43°C) was designated as the intracellular extract (IE). The method of Lowry *et al.* (1951) was used (with bovine serum albumin as standard) to determine protein content in the extracellular and intracellular extracts. Samples were also stored at -80°C for later SDS-PAGE analysis.

3.2.4.2 Detection and measurement of aminopeptidase activity

Aminopeptidase activity was measured using chromogenic substrates (p-nitroanilide derivatives of L-anomers of leucine, lysine, alanine, proline, arginine and methionine) according to the method of Fernandez-Espla *et al.* (1997). Aminopeptidase activity was assayed by incubation of 100µL of sample with 400µL of 50mM Tris-HCl buffer, pH 7, and 50µL of 10mM of substrate at 37°C for 20 minutes. The reaction was stopped by addition of 1mL of 30% acetic acid. The release of p-nitroanilide was followed by measuring the absorbance at 410nm. The concentration of p-nitroanilide was calculated from the derived value of molar absorption coefficient (ε = 9024mol/cm). One unit of enzyme activity was defined as the amount of enzyme required to release 1µmol of p-

nitroanilide per min under the above conditions of the assay. The specific activity was expressed as units per milligram of protein.

3.2.4.3 Detection and measurement of dipeptidase activity

Dipeptidase activity of the proteolytic strains of probiotic and yoghurt bacteria was measured using Ala-Met, Leu-Tyr, Leu-Gly, Ala-His, and Pro-Ile as substrates according to the method of Wohlrab and Bockelmann (1992). The reaction mixture contained 10μL of enzyme solution, 415μL of 50mM Tris-HCl, pH 7.5, 50μL of 22mM substrate, 25μL of peroxidase (5mg/mL in 0.8M $(NH_4)_2SO_4$), 25μL of L-amino acid oxidase (2mg/mL in distilled water), and 25μL of o-dianisidine (11.5mM), respectively. The test tubes containing reaction mixture were incubated at 50°C for 20 minutes. The reactions were stopped by the addition of 50μL of dithiothreitol (120mM). Oxidation of o-dianisidine coupled to substrate hydrolysis resulted in an increase of brown colour which was measured at 436nm. Enzyme activity was calculated by using a molar absorbance coefficient of 8100 mol/cm. The specific activity was expressed as units per milligram of protein.

3.2.4.4 Detection and measurement of endopeptidase and tripeptidase activity

Endopeptidase and tripeptidase activities in the EE and IE extracts were detected by thin-layer chromatography as per the method of Tan and Konings (1990). The reaction mixture contained 2mM substrate, 20mM Tris-HCl, pH 7, and an appropriate amount of extract. The reaction mixture was incubated for 60 minutes at 37°C. The reaction was stopped by adding 10μL of 30% acetic acid and the mixture was cooled to 43°C. Ten microlitres of the mixture was then spotted onto a precoated 0.25 cm-thick silica gel 60 plate (Merck, Darmstadt, Germany) and TLC was performed as described by Tan and Konings (1990). Peptides and amino acids became visible in UV light. Unless otherwise indicated, all experiments and analyses were replicated 3 times and the results presented are averages of the nine replicates.

3.2.5 Associative and inhibition studies in reconstituted skim milk

3.2.5.1 Investigation of yoghurt bacteria that stimulate and/or inhibit the growth of probiotic bacteria

This part of the study looked at the associative growth between selected yoghurt bacteria and probiotic bacteria according to the method of Santos *et al.* (1996). Each selected probiotic bacteria was inoculated into two 500mL Erlenmeyer flasks containing 200mL of RSM. One of the flasks was inoculated with the selected proteolytic yoghurt culture (1% v/v). The control and associative cultures were incubated (without shaking) for 48 hours at 37°C. Three millilitre samples were withdrawn from both flasks at intervals (0, 4, 8, 12, 24, and 48 hours) and determination of cfu/mL was carried out. Viable counts of yoghurt and probiotic bacteria were obtained by plating appropriate dilutions on appropriate media. Percentages of inhibition were calculated using the formula of Gilliland and Speck (1977):

$$\text{Percentage of inhibition} = \frac{(\text{cfu/mL in control}) - (\text{cfu/mL in associative culture})}{(\text{cfu/mL in control})} \times 100$$

Percentage of association = 100 – percentage of inhibition.

All experiments were carried out in duplicate

3.2.5.2 Detection and assay of inhibitory activity

Screening of inhibitory substance/s was carried out on solid media using a modification of the spot on the lawn method (Joseph *et al.* 1998). Agar (1%, 25mL) was poured in sterile petri plates, wells were cut in the agar using a 7mm sterile Wassermann tube and the bottom of the wells was sealed with 0.9% agar. Fifty microlitres of active culture of producer organism was filled into the well. The plates were left at room temperature for 2 hours to allow migration and settling of the test cultures and incubated for 3 hours at 37°C. After the initial growth, the remaining well was sealed with 1% agar. Finally, the spotted plates were overlaid with ~ 10mL of 0.9% agar seeded with 1% indicator organism containing 1-7 × 10^7 organisms/mL. The plates were allowed to solidify and

incubated for 24-48 hours at 37°C; aerobically for *S. thermophilus* and anaerobically for other organisms. After incubation, the plates were examined for zones of inhibition around the wells. Growth and inhibition studies of *S. thermophilus* were performed on ST agar devoid of bromocresol purple (Dave & Shah, 1996) and on MRS agar for the other groups of organisms.

3.2.6 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

3.2.6.1 Sample preparation

Whole bovine casein (250μg) (Sigma) was incubated in 50mM sodium phosphate buffer (pH 7.8; 200μL), at 37°C in the presence of 250 μg protein in the CWE, CFE and whole cell samples that were isolated from the selected bacterial cultures. The reaction was stopped at various time intervals (0, 2, 4, 6, and 8 hours) by addition of 12% TCA (200μL). After 15 minutes at room temperature, the reaction mixture was centrifuged. The pellet was resuspended in 100μL of distilled water. Protein content in samples was determined by the Lowry assay method using standard BSA.

3.2.6.2 SDS-PAGE method

SDS polyacrylamide gel electrophoresis was performed according to Laemmli (1970) using a Bio Rad mini-vertical electrophoresis system (Mini-Protean II Cell, Bio Rad Laboratories, Hercules, USA) with gels containing 12.5% acrylamide and bisacrylamide. Prestained SDS-PAGE molecular weight standards in the broad range of 6,800-198,000 daltons (Bio-Rad Laboratories) were used for calibration. Ten microlitres of protein samples were mixed with an equal volume of sample buffer and heated in a boiling water bath for 2-5 minutes. The samples were loaded in the wells of the SDS cast gels. Electrophoresis was carried out at 200V and 25mA for ~ 1 hour.

3.2.6.3 Development of SDS-PAGE gels

Casein fractions were detected by staining the 12.5% gels in 0.1% Coomassie brilliant blue solution for an hour while shaking, then destained in methanol, acetic acid and

water. Destaining solution was changed as often as necessary until the background of the destaining solution was clear.

3.2.6.4 Analysis of SDS-PAGE gels

Gels were dried using the BioRad gel air drying system (GelAir Cellophane Support 50 precut sheets) and analysed using the Luminescent Image Analyser (LAS-1000 plus, Fujifilm, Tokyo, Japan). Data was analysed with Image Gauge Software.

3.2.7 Capillary Electrophoresis

3.2.7.1 Sample preparation

Sample preparation was followed according to the method of Bockelmann *et al.* (1998). One mL protein extraction buffer (see 3.16.2) was added to 0.5g of milk. Milk proteins were completely dissolved by shaking for 90-120 minutes at 37°C in a shaking waterbath. Insoluble components and fat were separated from the soluble milk proteins by centrifugation (5 minutes, 14000 × g, 4°C). The fat layer was removed from the surface, and additional 500µL protein extraction buffer were added. After centrifugation (5 minutes, 14000 × g, 4°C) the supernatant was decanted and stored at -20°C.

3.2.7.2 Electrophoresis buffers

All buffers were prepared in milli-Q water and were filtered before use (0.2µm membrane filter, Millipore). For cleaning of capillaries, 0.1M NaOH was used and Milli-Q grade water was used for flushing before runs.

Electrophoresis buffer: A stock solution of 8M urea was prepared and stored frozen in aliquots at -20°C to avoid degradation. Ten millimolar tri-sodium citrate × $2H_2O$ and 0.05% (w/v) methylhydroxypropylcellulose (MHPC) were prepared in the urea stock solution; pH was adjusted to pH 2.5 with 2.5M citric acid; the final concentration of citric acid was about 0.5M and 6M for urea. Electrophoresis buffers were stored at 4°C for 4-6 weeks.

Protein extraction buffer: 5mM tri-sodium citrate × $2H_2O$ and 5mM dithiothreitol were dissolved in 6M urea, pH 8.0; the reducing buffer was stored in 2mL aliquots at -20°C and thawed immediately before use.

3.2.7.3 *Capillary electrophoresis protocol*

Capillary electrophoresis was carried out using a Beckman P/ACE system 2500 with Gold software data system version 810. Based on a method of De Jong *et al.* (1994) for the analysis of milk proteins, a CE method for the separation of caseins and corresponding proteolysis products was developed. For the separations, hydrophilic capillaries (CElect-P150, Supelco) were used. The length of the capillary was 50cm (30cm to the detector window) with an inner diameter of 50µm. The cathode buffer (10mL) was replaced weekly, the electrophoresis buffer, cleaning- and flushing solutions at the anode position (1.5mL each) were replaced daily.

Washing, flushing, and equilibration cycles were performed by manual injection. At the beginning of a daily series the capillary was cleaned and primed for the separations (2 minutes 0.1M NaOH, 4 minutes CE-grade water, 8 minutes electrophoresis buffer). The first run was always a blank run which was not used for analyses because of deviating peak areas and retention times.

For sample preparation, milk extracts were diluted with extraction buffer (1:5 v/v) and incubated for 30 minutes at room temperature. Electrophoresis was performed at 30°C using +25 kV for 23-28 minutes. The wavelength for detection was set to 212nm. Between runs, the capillary was flushed with CE-grade water for 0.2 minutes, and re-equilibrated for 4 minutes with CE buffer. When not in use, the capillary was stored in milli-Q grade water.

3.2.8 High performance liquid chromatography analysis of protein utilization patterns in LAB

3.2.8.1 Sample collection

Reconstituted skim milk was inoculated with each culture of *S. thermophilus* (2002, 2014, T-1, T-4, Y-1), *L. delbrueckii* ssp. *bulgaricus* (2501, 2515, Y-1), *L. acidophilus* (2405, 2415, T-1), *Bifidobacterium* ssp. (20210, 1941, 20099, T-1, BB-12), *S. thermophilus* 2002 + *L. delbrueckii* ssp. *bulgaricus* 2501, *L. acidophilus* 2405 + *Bifidobacterium* ssp. 1941, *S. thermophilus* 2014 + *L. delbrueckii* ssp. *bulgaricus* 2515, *L. acidophilus* 2415 + *B. thermophilum* 20210, *S. thermophilus* 2002 + *L. delbrueckii* ssp. *bulgaricus* 2501 + *L. acidophilus* 2415 + *B. thermophilum* 20210 and *S. thermophilus* 2014 + *L. delbrueckii* ssp. *bulgaricus* 2515 + *L. acidophilus* 2405 + *Bifidobacterium* ssp 1941. Incubation was carried out at 37°C. Samples were taken at 0, 6, 12 and 24 hours. Viable counts of cultures were determined in duplicate by pour plate method on appropriate media as outlined in section 3.2.2 in Chapter three. Ten millilitre of each sample was collected at each time interval and stored at -20°C until further analysis.

3.2.8.2 Sample preparation

A solution containing 0.1M BisTris buffer (pH 6.8), 6M guanidine HCl, 5.37mM sodium citrate, and 19.5mM DTT (pH 7) was added directly to 500µL aliquots of samples in a 1:1 ratio (v:v) at room temperature. After thawing, each sample was shaken for 10 seconds, incubated for 1 hour at room temperature, and centrifuged for 5 minutes at 16,000rpm in a micro centrifuge. The fat layer was then removed with a spatula. The remaining solubilized sample was diluted 1:3 (v:v) with a solution containing 4.5M guanidine-HCl and solvent A, which consisted of acetonitrile, water, and trifluoroacetic acid in a ratio 100:900:1 (v:v:v; pH 2). The concentration of milk protein in the final diluted solution was approximately 4mg/mL, whereas the concentration of milk protein in the original milk samples was usually between 30 and 33mg/mL.

3.2.8.3 Identification and quantification of milk proteins

For identification and quantification of milk proteins, a standard was prepared that contained purified bovine milk proteins (over 80% purity for κ–CN, over 85% purity for α_s-CN and α-LA, and over 90% purity for β-CN and β-LG according to Sigma). Concentrations in the standard were 1.5mg/mL κ-CN, 4.0mg/mL α_s-CN, 3.0mg/mL β-CN, 0.5mg/mL α-LA, and 1.0mg/mL β-LG. The standard was prepared as a single batch, and was frozen in aliquots at -20°C. Aliquots of standards and milk samples were treated the same.

3.2.9 Amino acid studies in LAB

3.2.9.1 Sample preparation

In Erlenmeyer flasks, 3g of overnight RSM cultures were suspended in 27g of 100mM citrate-HCl (pH 2.2) buffer. The mixture was moderately stirred for 20 minutes and filtered through a Schleicher & Schuell 595, half folded filter. Five grams of 3% (w/v) 5-sulfosalicylic acid were added to 1g of RSM filtrate and after 20 minutes of stirring, the suspension was filtered through a Schleicher & Schuell 595, half folded filter. One gram of filtrate was diluted (1:50) with 50mM borate-HCl (pH 9.0) buffer. Twenty microlitres of sample was used for HPLC analysis of free amino acids.

3.2.9.2 Amino acid supplementation studies

Reconstituted skim milk was inoculated with each culture of *S. thermophilus* 2002, *L. delbrueckii* ssp. *bulgaricus* 2501, *L. acidophilus* 2415, *B. thermophilum* 20210 and a mix culture of the previously mentioned to obtain initial populations of ~ 10^5-10^6cfu/mL. Amino acids including cysteine, methionine, threonine, alanine, glycine, aspartic acid, arginine, glutamic acid and cysteine + methionine were filter sterilized and added to give final concentration of 0.05% (w/v). Incubation was carried out at 37°C. Samples were taken at 0, 6, 12 and 24 hours. Development of acidity was determined by measuring the pH (at 0, 6, 12, and 24 hours) of bacterial culture by use of a standard pH meter.

3.2.10 Exopolysaccharide studies in LAB

3.2.10.1 Yoghurt preparation

The set yoghurts were manufactured using the culture collection cultures as outlined in Table 3.2. Homogenised and pasteurised milk (2L) was heated to 85°C for 30 minutes, cooled to 43°C and different combinations of bacterial cultures (1% of yoghurt mix) were added as shown in Table 3.1. The mix was distributed in 500mL (for firmness and viscosity studies) and 100mL plastic cups (for other analyses). Incubation was carried out at 42°C and fermentation was terminated at pH 4.5. After fermentation, yoghurt samples were removed and stored at 4°C for 4 weeks.

Table 3.2 The different combination of starter cultures used in yoghurt preparations.

Starter culture used for yoghurt production	Bacterial species
ABT[a]-1	ST[b]-1, LA[c]-1, and BB[d]-1
ABT-1 + LB[e] 2501	ST-1, LA-1, BB-1, and LB 2501
ABT-1 + LB 2515	ST-1, LA-1, BB-1, and LB 2515
ABT-4	ST-4, LA-4, and BB-4
ABT-4 + LB 2501	ST-4, LA-4, BB-4, and LB 2501
ABT-4 + LB 2515	ST-4, LA-4, BB-4, and LB 2515
Mix starter culture	ST 2002, LA 2415, and BB 20210
Mix starter culture+ LB 2501	ST 2002, LA 2415, BB 20210 and LB 2501
Mix starter culture+ LB 2515	ST 2002, LA 2415, BB 20210 and LB 2515

[a] ABT=*L. acidophilus*, and *Bifidobacterium* ssp. and *S. thermophilus.*
[b] ST=*S. thermophilus*
[c] LA=*L. acidophilus*
[d] BB=*Bifidobacterium* ssp.
[e] LB=*L. delbrueckii* ssp. *bulgaricus.*

3.2.10.2 Time interval specifications

The '0 day' (day 0) analyses were carried out after overnight cold storage of yoghurt samples, and 'week 1', 'week 2' and 'week 3' and 'week 4' analyses were carried out after 7, 14, and 21 and 28 days of storage, respectively.

3.2.10.3 Sample preparation

Yoghurt prepared in a 100mL yoghurt cup was taken at 0 day and for the rest of the storage period, where a sample was taken aseptically for microbiological analysis. Yoghurt prepared in 500mL cups was used for measuring the firmness of the product at day 0. After measuring the firmness, yoghurt sample was uniformly mixed and approximately 450mL sample taken in 500mL glass beakers for the measurement of viscosity. All the measurements were carried out in triplicate.

3.2.10.4 Texture analyses

The firmness of the set yoghurt at day 0 was measured with a cone penetrometer (Stanhope-Seta Ltd., Surrey, England). The first and second angles of the cone were at 30° and 90°, respectively, and the total weight of the cone with its holder was 102.52 g. The depth of cone penetration was measured in millimeters after 5 seconds of cone penetration. All analyses in triplicate were carried out at 4°C.

The apparent viscosity of yoghurt samples at 0 day was measured with a Brookfield viscometer (Model DV II, Brookfield Engineering Laboratory, Stoughton, USA) using a Helipath D spindle at 1.5rpm. Yoghurt samples were maintained at 4°C. Yoghurt, being a non-Newtonian fluid, gave no stable reading of viscosity, therefore the data presented are the averages of the highest and the lowest viscosity readings in centipoise obtained for each sample. The average range of fluctuations in viscosity readings was about 5-10%. For the viscosity measurements, a homogenously mixed yoghurt sample was filled in a 500mL glass beaker and the spindle allowed to spin in the sample.

3.2.10.5 Extraction and quantification of exopolysaccharide

Exopolysaccharide from yoghurts was extracted according to method of Uemura *et al.* (1998). The yoghurt sample (100g) was diluted twice with distilled water (200mL). Casein was precipitated by adjusting to pH 4.6 with 2M NaOH solution and removing it together with the microorganisms by centrifugation (10,000 × g, 4°C for 20 minutes). After neutralization of the whey fraction with the additional NaOH solution, the sample was heated for 30 minutes in a boiling water bath, and the insolubilized protein was

removed by centrifugation (10,000 × g, 4°C, 20 minutes). After the addition of an equal volume of cold ethanol (99.5%), the solution was stirred at 4°C overnight, and the resulting precipitate was recovered by centrifugation and then dissolved in distilled water. The precipitation procedure with ethanol was repeated twice. The separated crude polysaccharide was resuspended in 0.0M Tris-HCl buffer (pH 8.0) containing 1mM $MgCl_2$ and then treated with 2µg/mL of DNase and RNase each at 37°C, for 6 hours. The contaminating protein in the sample was digested with 0.2mg/mL of proteinase K overnight at 37°C. After stopping the reaction by using heat at 90°C for 10 minutes, the polysaccharide fraction was precipitated with ethanol and dialysed with distilled water, and then lyophilised (exocellular polysaccharide; EPS) and weighed. Total EPS was assayed and the quantification of sugar was carried out using the method of Dubois *et al.* (1956). The amount of EPS was expressed as microgram of glucose equivalents per gram of yoghurt using glucose as a standard.

3.2.11 Estimation of protein content

3.2.11.1 The Lowry Protein Assay

The Lowry protein assay was done according to the method described by Lowry *et al.* (1951). BSA (0.1%) was used as standard to construct the standard curve (0-100µg/µL). Solution A (0.5mL) is added to 0.5mL of each standard and unknown protein solution. This is further mixed and incubated at 37°C for 10 minutes. After ten minutes of incubation, 1.5mL of solution B is added to each standard and unknown, mixed and incubated at 52°C for 20 minutes. The absorbance for each standard and unknown protein solution is measured at 680nm using the spectrophotometer. A standard curve is then constructed for the values obtained for the BSA standards from which the protein concentration for the unknown samples is determined.

3.2.11.2 Coomassie-blue assay

The standard test tube assay procedure was used for determining protein concentration in the range of 100 to 1500µg/mL. A known protein concentration series was prepared by diluting a stock bovine serum albumin (BSA) standard in the same diluent as the protein

sample in which the concentration will be determined. The protein standard series used were 100, 250, 400, 500, 1000 and 1500μg/mL. Coomassie Protein Assay Reagent (5mL) was added to dilute standard BSA or unknown sample (0.1mL) and mixed. Sample diluent was used as "blank". Within 90 minutes, absorbance of standards and unknowns versus deionized water were read at 595nm. Protein concentration for each unknown protein sample was determined from BSA standard curve.

3.2.12 *Data handling and Statistical analysis*

Unless otherwise indicated, all experiments and analyses were replicated 3 times and results presented are averages of the nine replicates. The data was subjected to one-way ANOVA analysis (SigmaStat®, 1997).

Chapter 4 Proteolytic activity and proteolytic enzymes of yoghurt and probiotic bacteria*

4.1 Proteolytic activity of yoghurt and probiotic bacteria

4.1.1 Introduction

A rapid, simple, and accurate method to characterize proteolysis has been a goal of many dairy researchers. Such a method is needed in many applications, including starter culture technology, milk clotting chemistry, cheese aging, accelerated cheese ripening, shelf stabilization, microbial physiology, and microbial proteinase research. The Hull method (1947) relies on chemical characteristics of released tryptophan and tyrosine to reflect proteolysis. This method lacks sensitivity and specificity, especially in complex protein mixtures low in tryptophan or tyrosine. To reduce undesirable characteristics of the Hull method, the trinitrobenzenesulfonic acid (TNBS) procedure was developed. This method uses total soluble amino nitrogen to estimate proteolysis Other recently introduced methods to detect proteolysis include ^{14}C, spectrophotometric *0*-phthaldialdehyde (OPA), fluorometric OPA, laser fluorometry, reflectance colorimetry, fluorescamine, azocasein, casein agar, and resorufin-labeled casein (Weimer *et al.*, 1989).

Church *et al.* (1983) applied a method using OPA to determine proteolysis caused by lactic acid bacteria in milk. This procedure measures a culture's ability to produce primary amines from milk. Liberated primary amines, as amino acids, react with the OPA reagent, and the resulting color is measured with a spectrophotometer (Weimer *et al.*, 1989).

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Probiotic bacteria (*Lactobacillus acidophilus* and *Bifidobacterium* ssp.) grow slowly in milk because of lack of proteolytic activity (Klaver *et al.*, 1993), and the usual practice is to add yoghurt bacteria (*Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*) to probiotic products to reduce the fermentation time. *Lactobacillus delbrueckii* ssp. *bulgaricus* produces essential amino acids owing to its proteolytic nature, and the symbiotic relationship of *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus* is well established; the former organism produces amino nitrogen for the latter organism (Shihata & Shah, 2000).

In recent years, fermented milk products containing *L. acidophilus* and *Bifidobacterium* ssp. have been developed. There is an increasing interest in dairy products containing specific bacterial species with potential health benefits (Portier *et al.*, 1993). However, the slow growth of *L. acidophilus* and *Bifidobacterium* ssp. poses major difficulties for market expansion of probiotic products (Brasheras & Gilliland, 1995). Use of most proteolytic strains of *S. thermophilus*, *L. delbrueckii* ssp. *bulgaricus*, *L. acidophilus* and *Bifidobacterium* ssp. could enhance the growth and viability of probiotic bacteria in products over the storage period.

In this section, we present an evaluation of the proteolytic ability of various strains of *S. thermophilus*, *L. delbrueckii* ssp. *bulgaricus*, *L. acidophilus* and *Bifidobacterium* ssp.

4.1.2 Materials and methods

4.1.2.1 Bacterial cultures

The source and maintenance of pure cultures of 6 strains of *S. thermophilus*, 5 strains of *L. delbrueckii* ssp. *bulgaricus*, 13 strains of *L. acidophilus* and 12 strains of *Bifidobacterium* ssp. (*B. breve*, *B. infantis*, *B. adolescentis*, *B. longum*, *B. pseudolongum*, *B. thermophilum*, *Bifidobacterium* ssp., and *B. bifidum*), 3 strains of *S. thermophilus* (T-1, T-4, Y-1), one strain of *L. delbrueckii* ssp. *bulgaricus* (Y-2), and one strain each of *L. acidophilus* (B-1) and *Bifidobacterium spp* (B-2) is outlined and described in Chapter three, section 3.1.5 and 3.2.1 respectively.

4.1.2.2 Preparation of bacterial cultures for determination of proteolytic activity

Preparation of pure cultures of 6 strains of *S. thermophilus*, 5 strains of *L. delbrueckii* ssp. *bulgaricus*, 13 strains of *L. acidophilus* and 12 strains of *Bifidobacterium* ssp. *(B. breve, B. infantis, B. adolescentis, B. longum, B. pseudolongum, B. thermophilum, Bifidobacterium* ssp., and *B. bifidum*), 3 strains of *S. thermophilus* (T-1, T-4, Y-1), one strain of *L. delbrueckii* ssp. *bulgaricus* (Y-2), and one strain each of *L. acidophilus* (B-1) and *Bifidobacterium* spp (B-2) for the OPA assay is described and outlined in section 3.2.3.1 in Chapter three.

4.1.2.3 o-Phthaldialdehyde procedure

Quantification of free amino acids in pure cultures of 6 strains of *S. thermophilus*, 5 strains of *L. delbrueckii* ssp. *bulgaricus*, 13 strains of *L. acidophilus* and 12 strains of *Bifidobacterium* ssp. *(B. breve, B. infantis, B. adolescentis, B. longum, B. pseudolongum, B. thermophilum, Bifidobacterium* ssp., and *B. bifidum*), 3 strains of *S. thermophilus* (T-1, T-4, Y-1), one strain of *L. delbrueckii* ssp. *bulgaricus* (Y-2), and one strain each of *L. acidophilus* (B-1) and *Bifidobacterium* ssp. (B-2) was determined and measured according to the method of Church *et al.* (1983) as described in Chapter three, section 3.2.3.2.

4.1.2.4 Data handling and statistical analysis

The OPA procedure was replicated three times and final results are presented as averages of three replicates. The data was subjected to one-way ANOVA test (SigmaStat®, 1997).

4.1.3 Results and Discussion

The OPA-based spectrophotometric assay detects released α-amino groups, which result from the proteolysis of milk proteins, thus giving a direct measurement of proteolytic activity. Figs. 4.1.1 and 4.1.2 represent the proteolytic activities in RSM of 6 strains of *S. thermophilus*, 5 strains of *L. delbrueckii* ssp. *bulgaricus*, 12 strains of *L. acidophilus*, and 3 strains of *Bifidobacterium* ssp., respectively. The proteolytic activity of these bacterial

cultures is expressed as the amount of free amino groups measured as difference in absorbance values at 340nm, after subtraction of values for the uninoculated label control RSM. The extent of proteolysis based upon OPA values was variable between *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* strains (Fig. 4.1.1), *L. acidophilus* and *Bifidobacterium* strains (Figure 4.1.2) and appeared to be strain specific. These results have shown that the proteolytic activity of *S. thermophilus*, *L. delbrueckii* ssp. *bulgaricus*, and *L. acidophilus* was much higher than that of *Bifidobacterium* ssp., in agreement with Klaver *et al.* (1993), showing that *Bifidobacterium* strains are not highly proteolytic when compared to other lactic acid bacteria. Three *S. thermophilus* strains, one *L. delbrueckii* ssp. *bulgaricus* strain, two *L. acidophilus* strains and 10 *Bifidobacterium* strains did not show any proteolytic activity and hence those organisms are not represented in Figs. 4.1.1 and 4.1.2. *S. thermophilus* 2014 was highly proteolytic producing the highest amount of free amino groups (52.6μM) among the nine strains studied, whereas the strains T-1, T-4, and Y-1 were not proteolytic. The differences in the mean values among the *S. thermophilus* strains are greater than would be expected so the proteolytic activities among the *S. thermophilus* strains were significantly different ($P<0.001$).

L. delbrueckii ssp. *bulgaricus* strains were less proteolytic (Fig. 4.1.1) than *S. thermophilus* strains. *L. delbrueckii* ssp. *bulgaricus* strain 2515 showed the highest proteolytic activity releasing the highest amount of free amino groups (25μM). The proteolytic activities among the *L. delbrueckii* ssp. *bulgaricus* strains were significantly different ($P<0.001$). There was also a significant difference in proteolytic activity between the *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* ($P<0.001$) groups based on the amount of free amino groups released.

Starter cultures containing *L. acidophilus*, *Bifidobacterium* ssp., and *S. thermophilus* (ABT-cultures) are becoming popular in Australia. ABT-cultures lack *L. delbrueckii* ssp. *bulgaricus*, and as a result, the fermentation time for yoghurt making is longer with these cultures (Dave & Shah, 1997). Dave and Shah (1998) observed a 3-4 log cycle drop in the counts of *Bifidobacterium* in an ABT starter culture; supplementation with acid casein

hydrolysate, which contained peptides and amino acids, improved the viability of *Bifidobacterium* ssp. Among the probiotic bacteria, *L. acidophilus*, in general, was more proteolytic than *Bifidobacterium* ssp. (Fig. 4.1.2), and the differences in proteolytic activity between both groups were significantly different ($P<0.001$). These results agree with the findings of Singh and Sharma (1983) and Koroleva *et al.* (1983) who reported high proteolytic activity of selected strains of *L. acidophilus*. Among bifidobacteria, *B. thermophilum* 20210 released appreciable amounts of free amino groups (30μM). The rest of the strains did not release any amounts of free amino groups as compared to the control ($P<0.001$), thus those strains could be classified as non-proteolytic. This may explain why bifidobacteria grow slowly in milk. It is presumed that free amino acids could be utilised during the early stage of incubation and that peptides could become available during the prolonged incubation of *Bifidobacterium* cultures (Cheng & Nagasawa, 1984). This may also explain why the growth of bifidobacteria requires supplementation of peptides and amino acids from external sources (Dave & Shah, 1998). The differences in the mean values among all bacterial groups were significant ($P<0.001$) and greater than expected.

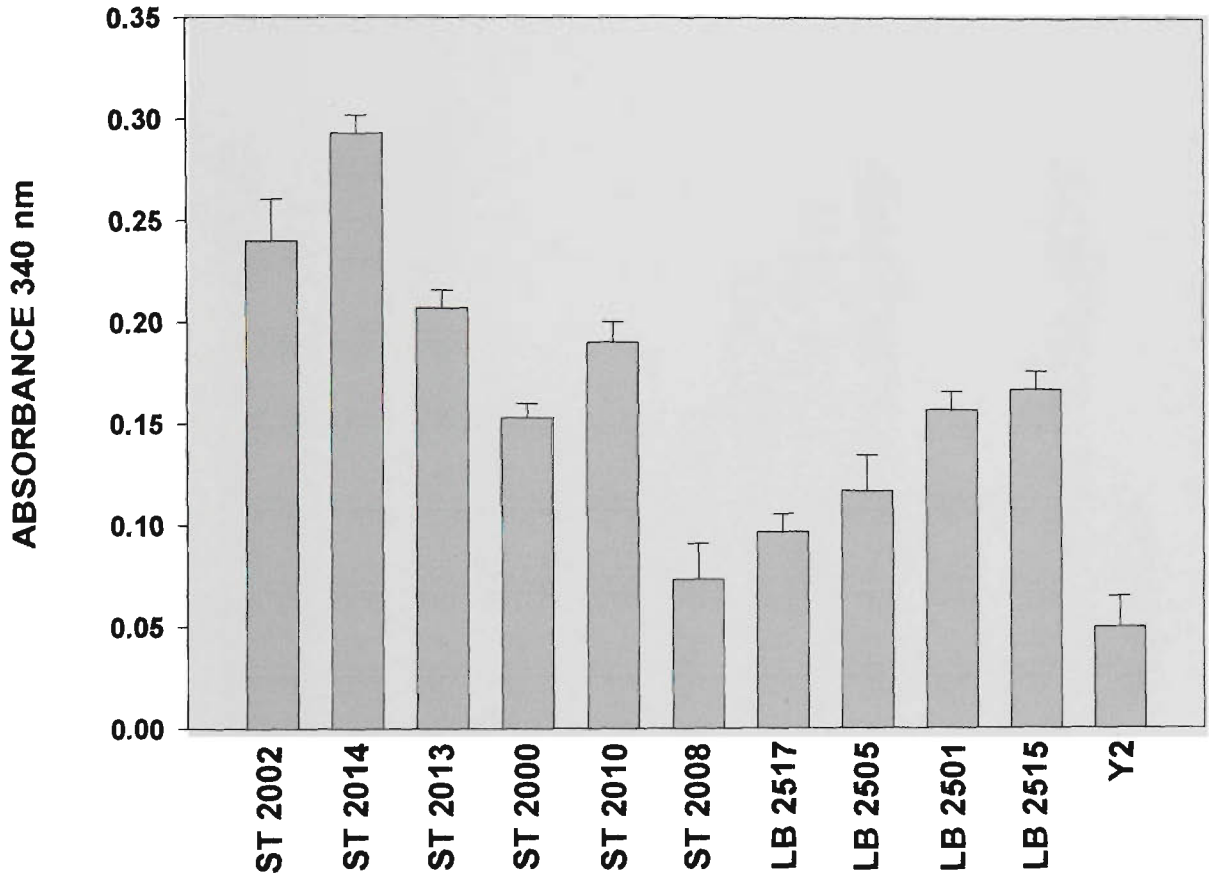

Figure 4.1.1 Indication of proteolytic activity of *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* strains after incubation in RSM at 37 and 42°C, respectively for 6 hours. Data represent differences in absorbance values after subtracting the value (1.68) for the control RSM.

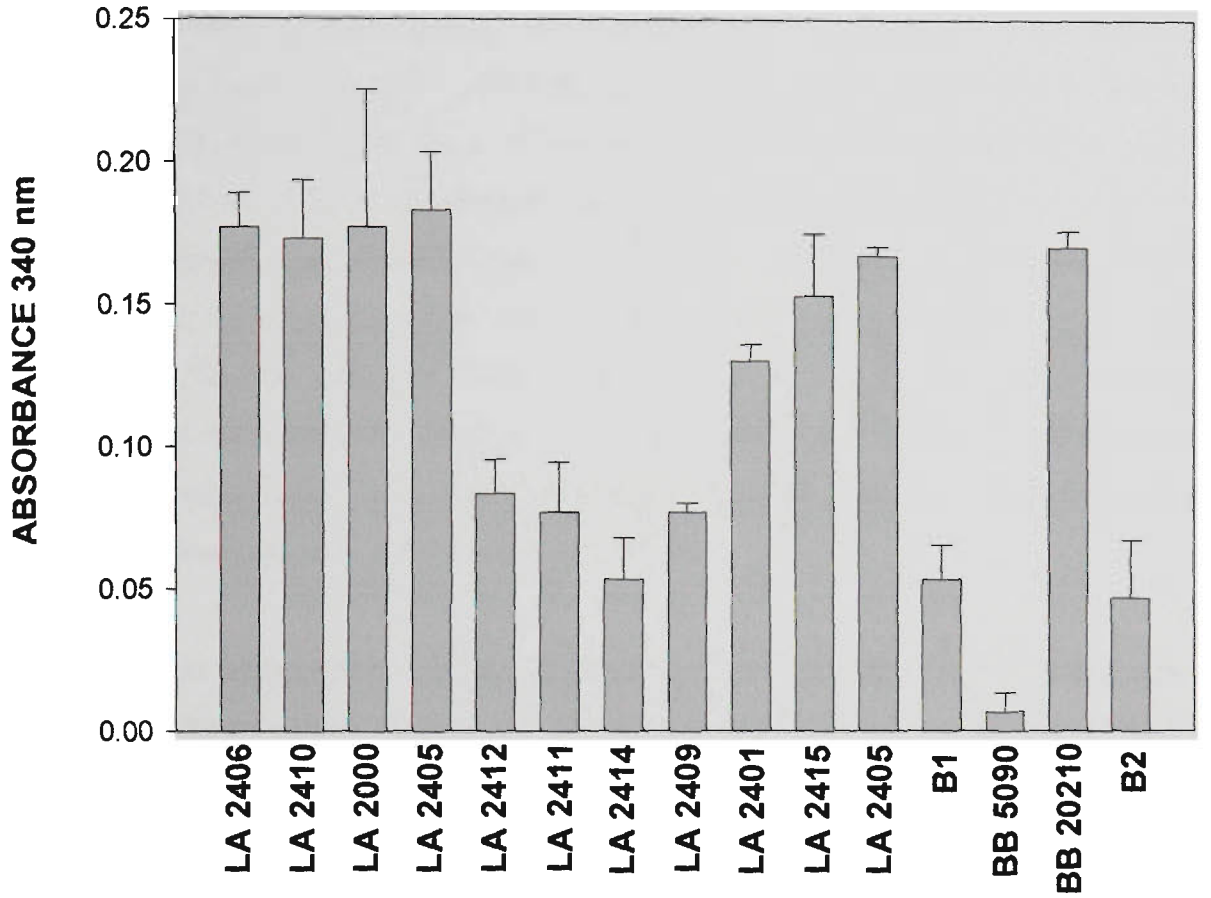

Figure 4.1.2 Indication of proteolytic activity of *L. acidophilus* and *Bifidobacterium* ssp. strains after incubation in RSM at 37°C for 6 hours. Data represent differences in absorbance values after subtracting the value (1.68) for the control RSM.

4.2 Proteolytic enzymes of yoghurt and probiotic bacteria

4.2.1 Introduction

It has been well-established that many lactic acid bacteria, isolated from milk products, are multiple amino acid auxotrophs. The requirement for amino acids is strain dependent and can vary from 4 up to 14 different amino acids. Lactic acid bacteria, therefore, depend for growth in milk on a proteolytic system that allows degradation of milk proteins (caseins). Caseins constitute about 80% of all proteins present in bovine milk. The four different types of caseins found in milk, αs1, αs2, β- and κ-casein, are organized in micelles to form soluble complexes. In free solution, caseins behave as non-compact and largely flexible molecules with a high proportion of residues accessible to the solvent. Caseins contain all amino acids necessary for growth of lactic acid bacteria in milk to high cell density, but it can be calculated that only a minor fraction of the total is actually needed (less than 1%) (Kunji *et al.*, 1996).

The structural components of the proteolytic systems of lactic acid bacteria can be divided into three groups on the basis of their function: (i) proteinases that breakdown caseins to peptides, (ii) peptidases that degrade peptides, and (iii) transport systems that translocate the breakdown products (small peptides and amino acids) across the cytoplasmic membrane. The proteinase is clearly involved in the initial degradation of caseins, yielding a large number of different oligopeptides. The initial analysis of the casein breakdown products liberated by the proteinases has indicated that, with a few exceptions, only large peptides are formed (Pritchard & Coolbear, 1993). Consequently, further breakdown by extracellular peptidases was considered to be critical to fulfill the needs for essential and growth-stimulating amino acids. The external localization of proteinases is consistent with the finding that these are synthesized with a typical signal peptide sequence, but this property has not been found in any of the peptidases analysed so far (Poolman *et al.* 1995). These findings are supported 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).

By far the most comprehensively understood proteolytic system of lactic acid bacteria is that of the mesophilic cheese starter strains of *Lactococcus lactis*. A model indicating possible pathways of proteolysis in lactococci are presented in Figure 4.2.1

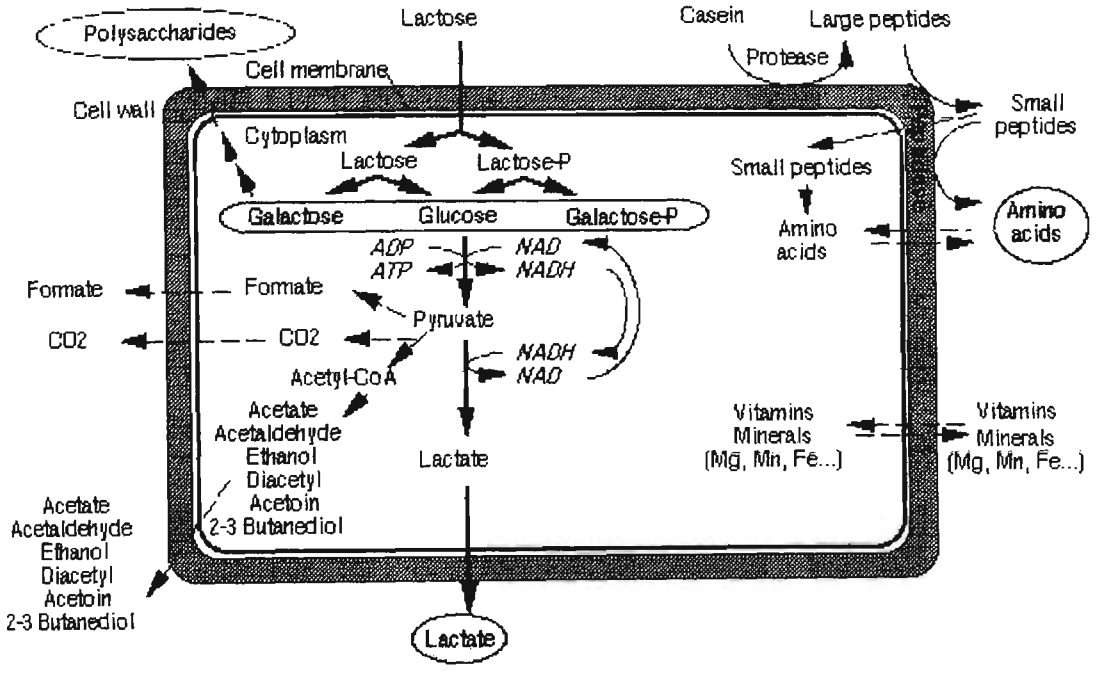

Figure 4.2.1 Possible pathways of proteolysis in lactococci (Anon, 1994).

The proteolytic activities of LAB including yoghurt bacteria and probiotic bacteria have been studied extensively and proteolytic enzymes have been isolated and characterised (Booth *et al.*, 1990; Wohlrab & Bockelmann, 1993; Bockelmann *et al.*, 1996; Law & Haandrikman, 1997). Endopeptidases hydrolyze large casein fragments into smaller peptides which can be degraded by several aminopeptidases. Specific X-prolyl-dipeptidylamino-peptidases degrade the proline-rich peptides while di- and tripeptidases and prolidases release free amino acids, which finally complete the degradation of casein (Tan *et al.*, 1991).

Several endopeptidases have been purified from yoghurt bacteria including *L. delbrueckii* ssp. *bulgaricus* B14 (Bockelmann *et al.*, 1996), and *S. thermophilus* CNRZ160 (Desmazeaud, 1974). Aminopeptidases are thought to be of ultimate importance for the

development of flavour in fermented milk products, since they are capable of releasing single amino acid residues from oligopeptides formed by extracellular proteinase activity. Metal-dependent aminopeptidases with a broad range specificity were characterised from *L. delbrueckii* ssp. *bulgaricus* B14 (Bockelmann *et al.*, 1995), *L. delbrueckii* ssp. *bulgaricus* (Atlan *et al.*, 1989), *L. acidophilus* R-26 (Machuga & Ives, 1984), *S. thermophilus* ACA-DC 114 (Tsakalidou & Kalantzopolous, 1992) and *S. thermophilus* CNRZ 302. *Bifidobacterium* ssp. is comparable to LAB by the presence of a general aminopeptidase activity encompassing several dipeptidases and possibly iminopeptidase and tripeptidases (Bockelmann & Fobker, 1991; Eggimann & Bachmann, 1980; Meyer & Jordi, 1987). Aminopeptidase and iminopeptidase activities were isolated from a cell-free extract of *B. breve* by Cheng and Nagasawa (1984, 1985). *B. infantis*, *B. longum*, and *B. adolescentis* showed aminopeptidase, dipeptidase, tripep-tidase and carboxypeptidase activities (El-Soda *et al.*, 1992). Dipeptidases have been purified from *S. thermophilus* (Rabier & Desmazeaud, 1973), and *L. delbrueckii* ssp. *bulgaricus* B14 (Wohlrab & Bockelmann, 1992) and tripeptidases from *L. delbrueckii* ssp. *bulgaricus* B14 (Bockelmann *et al.*, 1995; Argyle *et al.*, 1976) and *S. thermophilus* CNRZ160 (Rabier & Desmazeaud, 1973).

The proteolytic activity of yoghurt bacteria is weak, resulting in a breakdown of only 1-2% of milk protein (Rasic & Kurman, 1978). This proteolytic activity is necessary to release small peptides and amino acids for the growth of these bacteria. *L. delbrueckii* ssp. *bulgaricus* is more proteolytic, but both yoghurt bacteria contain peptidases which are necessary to hydrolyze large peptides into smaller peptides for transport into the cell. The principal substrate for proteolysis is casein, but limited degradation of whey proteins may also occur (Chandan *et al.*, 1982; Khalid *et al.*, 1991). The net effect of this proteolytic activity is that fermented milks have a higher content of peptides and free amino acids, especially valine, histidine, serine and proline than milk (Tamime & Deeth, 1980).

Several physical parameters that affect proteinase and peptidase activity in *L. delbrueckii* ssp. *bulgaricus* have been identified (Ezzat *et al.*, 1985; Frey *et al.*, 1986). Argyle *et al.*

(1976) characterized a cell-bound proteinase in *L. delbrueckii* ssp. *bulgaricus* NCDO 1489. Using the same organism, Chandan *et al.* (1982) examined its proteolytic ability on native micellar casein using the 2, 4, 6-trinitrobenzene sulfonic acid assay (TNBS) and noted that β-casein was the most susceptible fraction. Previously, Ohmiya and Sato (1978) had made this same observation, noting that intracellular proteases from *L. delbrueckii* ssp. *bulgaricus* and *Lactobacillus helveticus* hydrolyzed β-casein more readily then α_{s1}-casein. Ezzat *et al.* (1985 & 1982) found cell wall bound proteinase activity in *L. delbrueckii* ssp. *bulgaricus* by using ^{14}C methylated casein as substrate.

In recent years, fermented milk products containing *L. acidophilus* and *Bifidobacterium* ssp. have been developed. There is an increasing interest in dairy products containing specific bacterial species with potential health benefits (Portier *et al.*, 1993). However, the slow growth of *L. acidophilus* and *Bifidobacterium* ssp. poses major difficulties for market expansion of probiotic products (Brasheras & Gilliland, 1995). Use of most proteolytic strains of *S. thermophilus*, *L. delbrueckii* ssp. *bulgaricus*, *L. acidophilus* and *Bifidobacterium* ssp. could enhance the growth and viability of probiotic bacteria in products over the storage period. In this report, we present an evaluation of the peptidase profile of various strains of *S. thermophilus*, *L. delbrueckii* ssp. *bulgaricus*, *L. acidophilus* and *Bifidobacterium* ssp. Their peptidase profile including amino-, di-, tri- and endopeptidase activities are reported.

4.2.2 Materials and methods

4.2.2.1 Source and maintenance of bacterial cultures

Source and maintenance of 4 strains of *S. thermophilus* (2014, T-1, T-4, and Y-1), 2 strains of *L. delbrueckii* ssp. *bulgaricus* (2515 and Y-1), 2 strains of *L. acidophilus* (2405 and T-1) and 2 strains of *Bifidobacterium* ssp. (20210 and T-1) is described in Chapter three, section 3.1.5 and 3.2.1, respectively.

4.2.2.2 Preparation of intracellular and cell wall extracts

Intracellular and cell wall extracts of 4 strains of *S. thermophilus* (2014, T-1, T-4, and Y-1), 2 strains of *L. delbrueckii* ssp. *bulgaricus* (2515 and Y-1), 2 strains of *L. acidophilus* (2405 and T-1) and 2 strains of *Bifidobacterium* ssp. (20210 and T-1) were prepared according to the method described in section 3.2.4.1 in Chapter three.

4.2.2.3 Enzyme assays

4.2.2.3.1 Detection and measurement of aminopeptidase activity

Aminopeptidase activity in 4 strains of *S. thermophilus* (2014, T-1, T-4, and Y-1), 2 strains of *L. delbrueckii* ssp. *bulgaricus* (2515 and Y-1), 2 strains of *L. acidophilus* (2405 and T-1) and 2 strains of *Bifidobacterium* ssp. (20210 and T-1) was measured using the method described in section 3.2.4.2 in Chapter three.

4.2.2.3.2 Detection and measurement of dipeptidase activity

Dipeptidase activity in 4 strains of *S. thermophilus* (2014, T-1, T-4, and Y-1), 2 strains of *L. delbrueckii* ssp. *bulgaricus* (2515 and Y-1), 2 strains of *L. acidophilus* (2405 and T-1) and 2 strains of *Bifidobacterium* ssp. (20210 and T-1) was measured using the method described in section 3.2.4.3 in Chapter three.

4.2.2.3.3 Detection and measurement of endopeptidase and tripeptidase activity

Endopeptidase and tripeptidase activity in 4 strains of *S. thermophilus* (2014, T-1, T-4, and Y-1), 2 strains of *L. delbrueckii* ssp. *bulgaricus* (2515 and Y-1), 2 strains of *L. acidophilus* (2405 and T-1) and 2 strains of *Bifidobacterium* ssp. (20210 and T-1) was measured and detected according to the method described in section 3.2.4.4 in Chapter three.

4.2.2.4 Data handling and Statistical analysis

Enzymic analyses experiments were replicated three times and final results are presented as averages of the three replicates. The data was subjected to two-way ANOVA test (SigmaStat®, 1997)

4.2.3 Results and Discussion

4.2.3.1. Aminopeptidase activity

Selected strains of *S. thermophilus*, *L. delbrueckii* ssp. *bulgaricus*, *L. acidophilus*, and *Bifidobacterium* ssp. were further studied for their aminopeptidase, dipeptidase, tripeptidase, and endopeptidase activities, both at the extracellular (EE) and intracellular (IE) levels. *S. thermophilus* strains 2014, T-1, T-4, Y-1, *L. delbrueckii* ssp. *bulgaricus* strains 2515 and Y-2, *L. acidophilus* strains 2405 and B-1 and *B. thermophilum* 20210 and B-2 were selected for the enzyme assays. Aminopeptidase activity was detected for all bacterial strains, both at the EE and IE levels (Table 4.2.2). The specific activities towards the six substrates studied at the IE level were observed to be different to that at the EE level ($P<0.001$). Aminopeptidase activity at the IE level was the highest for *L. delbrueckii* ssp. *bulgaricus* followed by *Bifidobacterium* ssp., *S. thermophilus*, and *L. acidophilus*. *S. thermophilus* 2014 did not hydrolyse the substrate L-arginine-pNa ($P<0.05$). At the EE level, *L. delbrueckii* ssp. *bulgaricus* 2515 showed higher aminopeptidase activity than *L. delbrueckii* ssp. *bulgaricus* Y-2 strain. However, the opposite was true when comparing activities at the IE level except for L-arginine-pNa. L-proline-pNa was greatly hydrolyzed by *L. delbrueckii* ssp. *bulgaricus* (Y-2) at the IE level which indicates the presence of iminopeptidase activity. *L. acidophilus* strains showed higher activities at the IE level than the EE level. It is interesting to note that *L. acidophilus* 2405 showed greater specificity towards the substrate L-arginine-pNa at the EE level than at the IE level ($P<0.05$). Also, *Bifidobacterium* ssp. showed high levels of intracellular aminopeptidase activity but very little proteolytic activity. Desjardins *et al.* (1990) found that the number of α-amino groups released during the exponential and stationary phases of growth steadily increased, but the rate of proteolysis did not follow the rate of biomass production. *B. thermophilum* 20210 demonstrated higher

aminopeptidase activity both at the IE and EE levels ($P<0.05$) than *Bifidobacterium* strain B-2 which was isolated from a commercial starter culture. L-methionine was the preferred substrate by *B. thermophilum* 20210 followed by L-leucine, L-proline, L-alanine, L-lysine and L-arginine.

4.2.3.2 Dipeptidase activity

Dipeptidase activities of *S. thermophilus*, *L. delbrueckii* ssp. *bulgaricus*, *L. acidophilus*, and *Bifidobacterium* ssp. towards the five substrates selected are shown in Table 4.2.3. In general, *S. thermophilus* 2014 showed higher extracellular activity towards all five substrates studied, whereas the other strains showed variable specificity towards the substrates ($P<0.05$). *L. delbrueckii* ssp. *bulgaricus* showed higher dipeptidase activity, towards all substrates except for Leu-Gly when compared to strain Y-2 ($P<0.05$). Both lactobacilli, however, showed higher intracellular dipeptidase activity for four of the five substrates. Leu-Gly was greatly hydrolyzed by *S. thermophilus* T-4 at the EE level whereas the same applies for *L. delbrueckii* ssp. *bulgaricus* Y-1 at the IE level ($P<0.001$). *B. thermophilum* 20210 showed the highest activity towards the same substrates at the IE level. *L. acidophilus* 2405 showed similar activity when compared to the strain isolated from the commercial starter culture.

4.2.3.3 Endo- and tripeptidase activity

All bacterial cultures tested were able to hydrolyse the large biologically active peptides, bradykinin and Ala-Ala-Ala-Ala-Ala at both the extracellular and intracellular levels. The same can be deduced for the tripeptide substrate Gly-Ala-Tyr (Table 4.2.4). However, with the substrates ending with a C-terminal of phenylalanine, Gly-Leu-Phe, and Gly-Gly-Phe, the hydrolysis only occurred at the IE level. This suggests the presence of a tripeptidase at the IE level.

4.2.4 Conclusions

The yoghurt bacteria *(S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus*) appeared to be highly proteolytic as compared to the probiotic bacteria *(L. acidophilus* and *Bifidobacterium* ssp.). The yoghurt bacteria released higher amounts of free amino acids

and demonstrated greater aminopeptidase and dipeptidyl activity than the probiotic bacteria. As a result, yoghurt bacteria grow faster in milk, whereas the probiotic bacteria grow slowly due to lack of proteolytic activity and require an exogenous supply of peptides and amino acids for optimum growth, in particular for starter cultures that do not contain *L. delbrueckii* ssp. *bulgaricus*.

Table 4.2.2 Aminopeptidase activity[a] of *S. thermophilus*, *L. delbrueckii* ssp. *bulgaricus*, *L. acidophilus* and *Bifidobacterium* ssp.

Bacterial cultures	SUBSTRATES[b]					
	Leu-pNa	Lys-pNa	Pro-pNa	Ala-pNa	Meth-pNa	Arg-pNa
***S. thermophilus* 2014**						
EE[c]	4.55 ± 6.36[d]	6.67 ± 0.30	10.61 ± 0.61	20.91 ± 2.10	16.06 ± 2.42	0.00 ± 0.00
IE[e]	27.04 ± 0.45	35.67 ± 0.46	17.89 ± 0.00	18.48 ± 1.47	29.33 ± 0.94	132.91 ± 7.82
***S. thermophilus* ABT-1**						
EE	2.43 ± 0.37	4.83 ± 0.07	6.43 ± 0.09	3.87 ± 0.14	4.30 ± 0.44	4.77 ± 0.07
IE	6.07 ± 0.52	5.83 ± 1.74	7.37 ± 0.65	11.80 ± 1.04	7.63 ± 1.16	10.37 ± 0.61
***S. thermophilus* ABT-4**						
EE	2.90 ± 0.00	6.17 ± 0.18	5.63 ± 0.77	5.30 ± 0.19	4.83 ± 0.35	5.97 ± 0.37
IE	18.23 ± 1.81	19.77 ± 1.58	12.23 ± 0.23	19.33 ± 0.38	12.87 ± 0.81	21.77 ± 0.79
***S. thermophilus* ABY-1**						
EE	3.27 ± 0.28	3.17 ± 0.07	5.30 ± 0.40	4.57 ± 0.10	4.50 ± 0.20	4.47 ± 0.03
IE	6.57 ± 0.50	3.53 ± 0.59	5.93 ± 0.24	7.97 ± 0.24	5.27 ± 0.27	8.17 ± 0.23
***L. bulgaricus* 2515**						
EE	15.78 ± 1.00	9.39 ± 1.59	10.27 ± 0.27	19.46 ± 0.18	21.77 ± 0.27	148.14 ± 1.43
IE	84.04 ± 2.75	129.76 ± 3.33	39.29 ± 1.80	46.90 ± 1.67	56.90 ± 3.92	185.13 ± 5.88
***L. bulgaricus* ABY-1**						
EE	5.07 ± 0.08	3.68 ± 0.28	4.17 ± 0.21	6.79 ± 0.50	3.76 ± 0.29	4.25 ± 0.09
IE	129.67 ± 5.21	320.61 ± 16.31	293.73 ± 0.58	88.21 ± 5.23	75.06 ± 2.30	13.51 ± 1.58
***L. acidophilus* 2405**						
EE	7.94 ± 0.34	5.96 ± 0.28	5.98 ± 0.32	6.94 ± 0.02	8.21 ± 0.11	43.50 ± 1.72
IE	21.40 ± 0.94	24.50 ± 1.37	15.81 ± 0.70	15.81 ± 0.48	21.24 ± 0.89	8.30 ± 0.26
***L. acidophilus* ABT-1**						
EE	5.43 ± 0.10	7.00 ± 0.06	7.70 ± 0.66	6.60 ± 0.17	5.97 ± 0.07	6.70 ± 0.31
IE	21.43 ± 0.33	47.00 ± 3.51	13.33 ± 1.54	21.77 ± 0.72	14.20 ± 1.10	36.00 ± 1.85
***Bifidobacterium* ssp.20210**						
EE	9.36 ± 0.06	7.67 ± 0.42	7.83 ± 0.29	8.13 ± 0.02	8.45 ± 0.18	328.27 ± 0.64
IE	94.33 ± 1.45	66.67 ± 1.45	87.33 ± 4.84	81.00 ± 0.58	98.33 ± 1.10	8.65 ± 0.09
***Bifidobacterium* ssp. ABT-1**						
EE	2.6 ± 0.10	4.97 ± 0.07	4.93 ± 0.09	4.37 ± 0.10	4.10 ± 0.10	4.57 ± 0.03
IE	10.67 ± 0.13	9.07 ± 0.35	10.00 ± 0.46	11.60 ± 0.46	10.67 ± 1.33	11.53 ± 1.41

[a] Aminopeptidase activity expressed as specific activity which is defined as units of enzyme activity per milligram of protein.
[b] Leu-pNa, Lys-pNa, Pro-pNa, Ala-pNa, Meth-pNa and Arg-pNa are p-nitroanilide derivatives of these amino acids.
[c] EE = cell wall extracellular extract.
[d] Values are means ± standard error
[e] IE = intracellular extracts

Table 4.2.3 Dipeptidase activity[a] of *S. thermophilus*, *L. delbrueckii* ssp. *bulgaricus*, *L. acidophilus* and *Bifidobacterium* ssp.

Bacterial cultures	Substrates				
	Ala-Met	*Leu-Tyr*	*Pro-Ile*	*Leu-Gly*	*Ala-His*
***S. thermophilus* 2014**					
EE[b]	1100 ± 6.41[c]	992.73 ± 6.88	948.48 ± 13.21	7.86 ± 0.73	576.00 ± 11.65
IE[d]	94.22 ± 1.63	120.44 ± 9.77	14.81 ± 0.53	3.94 ± 0.29	11.79 ± 0.78
***S. thermophilus* ABT-1**					
EE	28.40 ± 1.90	26.93 ± 1.68	61.93 ± 2.99	17.02 ± 1.02	13.83 ± 2.24
IE	60.00 ± 3.13	56.90 ± 3.25	10.97 ± 0.99	13.83 ± 2.24	33.95 ± 0.63
***S. thermophilus* ABT-4**					
EE	30.73 ± 1.85	35.90 ± 0.46	86.53 ± 0.83	18.84 ± 0.55	22.89 ± 0.97
IE	91.77 ± 5.03	97.10 ± 4.83	13.10 ± 0.20	22.89 ± 0.97	37.78 ± 2.74
***S. thermophilus* ABY-1**					
EE	20.57 ± 0.23	30.23 ± 1.98	47.57 ± 4.42	15.97 ± 0.76	11.25 ± 0.44
IE	24.20 ± 1.84	59.90 ± 0.55	5.37 ± 0.52	11.25 ± 0.44	12.34 ± 0.16
***L. bulgaricus* 2515**					
EE	173.73 ± 5.59	154.27 ± 8.75	182.40 ± 5.31	1.40 ± 0.09	97.72 ± 2.23
IE	1270.48 ± 8.66	884.76±11.38	135.71 ± 1.65	2.43 ± 0.09	438.29 ± 0.41
***L. bulgaricus* ABY-1**					
EE	8.96 ± 0.42	8.57 ± 1.24	8.99 ± 0.96	6.49 ± 0.55	58.46 ± 0.99
IE	9.30 ± 0.43	8.88 ± 0.81	38.06 ± 0.88	58.58 ± 0.88	63.97 ± 1.70
***L. acidophilus* 2405**					
EE	46.37 ± 1.82	43.63 ± 1.50	49.80 ± 0.98	0.46 ± 0.03	30.05 ± 0.95
IE	126.67 ± 8.24	105.76 ± 2.81	22.88 ± 0.66	0.82 ± 0.03	106.11 ± 1.19
***L. acidophilus* ABT-1**					
EE	18.57 ± 1.40	17.33 ± 1.43	39.30 ± 0.46	7.60 ± 0.33	23.58 ± 1.00
IE	36.83 ± 7.53	84.73 ± 0.32	15.27 ± 1.57	23.58 ± 1.00	26.19 ± 1.09
***Bifidobacterium* spp 20210**					
EE	41.10 ± 1.11	40.59 ± 0.91	37.95 ± 3.25	0.48 ± 0.04	20.60 ± 1.96
IE	1304.00 ± 8.33	1074.00 ± 9.45	90.00 ± 4.16	2.67 ± 0.14	141.20 ± 6.80
***Bifidobacterium* spp ABT-1**					
EE	12.57 ± 0.09	11.67 ± 0.52	25.63 ± 0.23	5.08 ± 0.27	13.04 ± 0.31
IE	28.40 ± 2.20	37.73 ± 3.93	9.87 ± 0.53	13.04 ± 0.31	12.79 ± 0.30

[a] Dipeptidase activity expressed as specific activity which is defined as units of enzyme activity per milligram of protein.
[b] EE = extracellular cell wall extract
[c] Values are means ± standard error
[d] IE = intracellular extract

Table 4.2.4 Tripeptidase and endopeptidase activity[a] of *S. thermophilus*, *L. delbrueckii* ssp. *bulgaricus*, *L. acidophilus* and *Bifidobacterium* ssp.

	Hydrolysis[b]	
Substrates	CWE[c]	IE[d]
Tripeptides		
Gly-Ala-Tyr	+	+
Gly-Leu-Phe	–	+
Gly-Gly-Phe	–	+
Endopeptides		
Bradykinin	+	+
Ala-Ala-Ala-Ala-Ala	+	+

[a] Hydrolysis of peptides was analyzed by TLC
[b] + = Hydrolysis; – = no hydrolysis
[c] CWE = cell wall extract
[d] IE = intracellular extract

Chapter 5 Associative and/or inhibitory growth between yoghurt and probiotic bacteria

5.1 Introduction

The fermentation of milk to make yoghurt is due to the growth in association of *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus*. The interaction between these two types of bacteria, termed protocooperation, is beneficial but not obligatory for their growth. *L. delbrueckii* ssp. *bulgaricus* is more proteolytic than *S. thermophilus* and consequently produces small peptides for the latter, while *S. thermophilus* produces formic acid, necessary for purine biosynthesis in *L. delbrueckii* ssp. *bulgaricus*. Decarboxylation of urea by ureases which are present in most strains of *S. thermophilus* yields CO_2, which is involved in aspartate biosynthesis by *L. delbrueckii* ssp. *bulgaricus* (Tamime & Deeth, 1980).

The observation of a symbiotic relationship between *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* in the yoghurt starter culture was first reported by Orla-Jensen (1919). Pette and Lolkema (1950a) observed more rapid acid development in mixed cultures due to the increase in number of streptococci. They also demonstrated active growth of *S. thermophilus* in milk containing *L. delbrueckii* ssp. *bulgaricus* milk filtrate, and concluded that *L. delbrueckii* ssp. *bulgaricus* provided essential growth requirements for stimulation of *S. thermophilus* (Tamime & Deeth, 1980).

Bautista *et al.* (1966) confirmed that *L. delbrueckii* ssp. *bulgaricus* stimulates *S. thermophilus* and concluded that glycine and histidine are the essential amino acids; they failed to observed stimulation by valine. However, Accolas *et al.* (1971) observed stimulation of *S. thermophilus* by various mixtures of valine, leucine, histidine and isoleucine. Braquart *et al.* (1978a & b) concluded that omission of glutamic acid, valine, leucine, histidine and tryptophan from the growth medium reduced the stimulation by 50%. Similar findings were reported by Shankar (1977), Shankar and Davies (1977) and Higashio *et al.* (1977a). Galesloot *et al.* (1968) investigated the opposite side of the relationship, and reached the conclusion that *S. thermophilus*, under anaerobic conditions,

produces a stimulatory substance for *L. delbrueckii* ssp. *bulgaricus* that is equal to, or can be replaced by, formic acid. Veringa *et al.* (1968) confirmed that the stimulatory factor reported by Galesloot *et al.* (1968) was formic acid, and that it was produced by *S. thermophilus* (Tamime & Deeth, 1980).

Other compounds found to be stimulatory to *S. thermophilus* are peptides containing lysine resulting from hydrolysis of casein by *Micrococcus caseolyticus* (Desmazeaud & Hermier, 1972) or hepta- and pentapeptides containing histidine obtained by hydrolysis of glucagons by papain (Desmazeaud & Hermier, 1973). Lower peptides and free non-aromatic amino acids resulting from enzymic digestion of casein or trypsin-hydrolysed casein were also stimulatory to *S. thermophilus*. *L. delbrueckii* ssp. *bulgaricus* can be stimulated by purine, adenine, guanine, uracil and adenosine (Tamime & Deeth, 1980).

It can be seen from the above findings that the symbiotic behaviour of the yoghurt starter culture is important. At some stage during the incubation period the *L. delbrueckii* ssp. *bulgaricus* provides the essential amino acids required by *S. thermophilus* and the latter provides formic acid-like compounds stimulatory by peptides, purines or pyrimidines during yoghurt production. It was also demonstrated that *S. thermophilus* can inhibit *L. delbrueckii* ssp. *bulgaricus* in the exponential and stationary phase due to limited nutrient in the medium, because the former organism is a better competitor that *L. delbrueckii* ssp. *bulgaricus*. However, this observation has not been reported by other workers (Tamime & Deeth, 1980).

In mixed cultures, it is necessary to use strains which do not negatively influence the growth of each other. In this respect it is important to investigate the inhibitory activity of the starter components (Ivanova *et al.*, 1998). Therefore, it can be concluded that the relationship between *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* is mostly

symbiotic; however, not much is known about the nature of the relationship between yoghurt bacteria and probiotic bacteria in a mixed culture.

Dave and Shah (1997a) have reported the counts of bifidobacteria to dramatically decrease from their initial numbers in one of the four commercial starter cultures evaluated. The inhibition was not due to organic acids or hydrogen peroxide (Dave & Shah, 1997a). Increased inoculum (Dave & Shah, 1997b) or use of ascorbic acid as an oxygen scavenger (Dave & Shah, 1997c) did not improve viability of bifidobacteria in yoghurt made with this commercial starter culture. Therefore, inhibition of this organism was presumed to be due to antagonism among starter bacteria. The information on antagonism effects due to bacteriocins between yoghurt bacteria (*S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus*) and probiotic bacteria (*L. acidophilus* and bifidobacteria) is scant. Joseph *et al.* (1997) reported that *L. acidophilus* strains produced a substance active against strains of *L. delbrueckii* ssp. *bulgaricus*.

Bacteriocins are proteinaceous compounds that kill or inhibit closely related bacteria. However, research has shown that bacteriocins may exhibit bacteriocidal activity beyond species that are closely related. Various organisms belonging to species such as *Lactococcus*, *Pediococcus*, *Lactobacillus*, *Leuconostoc*, *Carnobacterium*, *Propionobacterium*, *Enterococcus*, *Bacillus* and *Escherichia* have been reported to produce bacteriocins or bacteriocin-like inhibitory substances (Dave & Shah, 1997).

In this study, the nature of the relationship between probiotic bacteria and yoghurt bacteria was investigated. The aim was to find out whether the relationship between the two groups of microorganisms was an associative and/or inhibitory one. This was carried out by growing the organisms together and determining the log viable counts over 24 hour incubation period and screening these organisms for inhibitory activity against each other.

5.2 Materials and Methods

5.2.1 Source and maintenance of bacterial cultures

The source and maintenance of *S. thermophilus* (2002 and 2014), *L. delbrueckii* ssp. *bulgaricus* (2501 and 2515), *L. acidophilus* (2405 and 2415), *B. thermophilum* 20210, *B. pseudolongum* 20099, and *B. longum* 1941 is described in detail in Chapter three in section 3.1.5 and 3.2.1 respectively.

5.2.2 Detection of inhibitory activity

Screening of inhibitory substance/s by yoghurt bacteria, *S. thermophilus* (2002 and 2014) and *L. delbrueckii* ssp. *bulgaricus* (2501 and 2515) and probiotic bacteria, *L. acidophilus* (2405 and 2415), *B. thermophilum* 20210, *B. pseudolongum* 20099, and *B. longum* 1941 was carried out on solid media using a modification of the spot on the lawn method (Joseph *et al.* 1998) as described in detail in section 3.2.5.2 in Chapter three. These screenings tests were duplicated.

5.2.3 Associative and/or inhibitory growth of yoghurt bacteria with probiotic bacteria in MRS broth

The nature of the relationship between yoghurt bacteria (*S. thermophilus* 2002, *S. thermophilus* 2014, *L. delbrueckii* ssp. *bulgaricus* 2501 and *L. delbrueckii* ssp. *bulgaricus* 2515) and probiotic bacteria (*L. acidophilus* 2405, *L. acidophilus* 2415, *B. thermophilum* 20210, *B. pseudolongum* 20099, and *B. longum* 1941) when in co-culture was investigated using the method of Santos *et al.* (1996) as described in detail in section 3.2.5.1 in Chapter three. Viable counts of yoghurt and probiotic bacteria were obtained by pour plating appropriate dilutions on appropriate media as described in detail in section 3.2.2 in Chapter three. Percentages of inhibition were calculated using the formula of Gilliland and Speck (1977):

$$\text{Percentage of inhibition} = \frac{(\text{cfu/mL in control}) - (\text{cfu/mL in associative culture})}{(\text{cfu/mL in control})} \times 100$$

Percentage association = 100 – percentage inhibition

All experiments were carried out in duplicate. Final results are presented as averages of duplicates. The data was subjected to one-way ANOVA analysis (SigmaStat®, 1997).

5.3 Results and Discussion

In this study, specific *S. thermophilus* (ST), *L. delbrueckii* ssp. *bulgaricus* (LB), *L. acidophilus* (LA) and *Bifidobacterium* ssp. (BB) strains were selected based on earlier studies that have shown that *S. thermophilus* (2002 and 2014), *L. delbrueckii* ssp. *bulgaricus* (2501 and 2515), *L. acidophilus* (2405 and 2415), *B. thermophilum* 20210 were highly proteolytic and were able to release higher amounts of free amino acids (Shihata & Shah, 2000). *B. longum* 1941 and *B. pseudolongum* 20099 were selected on the basis of their ability to tolerate high levels of acid and bile (Lankaputhra & Shah, 1995). This characteristic places these two strains high up in the selection category for use as a probiotic, thus they were introduced in this study.

All the nine bacterial strains were screened against the same nine-strain panel as indicator organisms. Inhibition of *S. thermophilus*, *L. delbrueckii* ssp. *bulgaricus*, *L. acidophilus*, or *Bifidobacterium* ssp. strains was not observed by any of the *S. thermophilus* or *L. delbrueckii* ssp. *bulgaricus* strains. *S. thermophilus* 2002, *L. delbrueckii* ssp. *bulgaricus* 2501, and *L. acidophilus* 2415 strain were inhibited by *L. acidophilus* 2405 strains. These inhibitions were observed during initial screening with modified spot on lawn technique, but not observed with the agar well assay technique. Inhibition of *S. thermophilus* (2002 and 2014), *L. delbrueckii* ssp. *bulgaricus* (2501 and 2515), and *L. acidophilus* (2415) by *L. acidophilus* 2405 strains is given in Table 5.1. These are the results of inhibition zones that were well-defined during the initial screening of cultures using modified spot on lawn technique. The inhibition caused by the producer organisms could have been due to H_2O_2, organic acids or to a bacteriocin-like inhibitory substance (BLIS) which has been investigated and shown in previous studies (Joseph *et al.*, 1998).

Inhibition of *S. thermophilus* 2002 by *L. acidophilus* 2405 was observed. *S. thermophilus* 2014 was not inhibited by any of the bacterial strains tested. Inhibition of *L. delbrueckii* ssp. *bulgaricus* 2501 was also observed by *L. acidophilus* 2415. Inhibitory activity of *L. acidophilus* strains was lost when the MRS broth was treated with proteolytic enzymes, chymotrypsin and papain. This confirmed that an active protein moiety could be involved in the inhibition of *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* strains. These findings agree with those found earlier by Joseph *et al.* (1998).

Thus, it could be concluded that *L. acidophilus* strains (2405 and 2415) could be producing a bacteriocin-like inhibitory substance (BLIS) which was active against *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus* strains. As stated earlier, Dave and Shah (1997a, b) reported that a strain of bifidobacteria lost its viability in yoghurts made with commercial starter cultures that contained yoghurt bacteria and probiotic bacteria. Such inhibition could be due to the production of antimicrobial substances in yoghurt by associative organisms; though this was not observed in this study. *S. thermophilus* and some *Bifidobacterium* ssp. have been reported to be sensitive to low pH (Davis *et al.*, 1971; Martin & Chou, 1992; Lankaputhra *et al.*, 1996). The inhibition observed for *S. thermophilus* 2002 could be due to its sensitivity to acid.

The precise mechanism by which LAB cause inhibition of microorganisms appears to be rather complex and has not been fully understood. The inhibitory activity of LAB can be attributed to creation of a hostile environment for foodborne pathogens and spoilage organisms in foods. Mechanisms proposed for such effects include production of lactic acid and other organic acids, hydrogen peroxide, competition and nutrient depletion, altered oxidation-reduction potential, and production of bacteriocins or antibiotics. However, it is generally believed that the inhibitory activity is a composite effect of several factors (Shah, 2000).

Therefore, based on the results of the modified spot on lawn method, *S. thermophilus* 2002, *L. delbrueckii* ssp. *bulgaricus* 2501, *L. delbrueckii* ssp. *bulgaricus* 2515, *L. acidophilus* 2405 and *B. thermophilum* 20210 were selected for further studies. The main

objective of these investigations was to study the nature of the relationship between yoghurt bacteria and probiotic bacteria in co-culture, i.e. whether the relationship is an antagonistic or inhibitory one. Figure 5.1 shows log viable counts of *B. thermophilum* 20210 in single culture, *B. thermophilum* 20210 and *S. thermophilus* 2002 in co-culture over a 24 hour incubation period in MRS broth at 37°C. Growth of *B. thermophilum* 20210 did not improve when in co-culture with *S. thermophilus* 2002. *B. thermophilum* 20210 survived better alone than when co-cultured with *S. thermophilus* 2002. The viable counts for *B. thermophilum* 20210 were 2 logs better when grown alone than when in co-culture with *S. thermophilus* 2002 (Figure 5.1). This could be because *S. thermophilus* strains have been reported to be highly competitive for nutrients and highly proteolytic (Shihata & Shah, 2000). As nutrients are already low in MRS broth, *S. thermophilus* 2002 would naturally out-compete *B. thermophilum* 20210 for available nutrients because *S. thermophilus* 2002 grows faster than *B. thermophilum* 20210. At 8 hours, there was a 28% association observed between *S. thermophilus* 2002 and *B. thermophilum* 20210. *S. thermophilus* 2002 was inhibitory (71%) to the growth of *B. thermophilum* 20210 (Figure 5.7).

Figure 5.2 represents the log viable counts of *B. thermophilum* 20210 in single culture, *B. thermophilum* 20210, and *L. delbrueckii* ssp. *bulgaricus* 2501 in co-culture during a 24 hour incubation period in MRS broth at 37°C. *B. thermophilum* 20210 survived better alone than when co-cultured with *L. delbrueckii* ssp. *bulgaricus* 2501. The relationship between *B. thermophilum* 20210 and *L. delbrueckii* ssp. *bulgaricus* 2501 appeared to be an associative one at 0 and 4 hours, where the antagonism between both bacterial species was 57% and 58%, respectively (Figure 5.7). The percentage association between the two organisms decreased significantly ($P<0.001$) at 8, 12, and 24 hours (Figure 5.7). This could be attributed to the highly proteolytic nature of *L. delbrueckii* ssp. *bulgaricus* 2501 (Shihata & Shah, 2000).

Previous studies conducted by Shihata and Shah (2000) have also shown that *L. delbrueckii* ssp. *bulgaricus* 2501 demonstrated greater peptidase activity where it has the ability to degrade essential substrates in the growth medium to produce essential peptides

and amino acids that could have had an effect on the initial viable counts of *B. thermophilum* 20210, i.e. 0 and 4 hours (Figure 5.7). However, there was a 2-log difference in viable counts between *B. thermophilum* 20210 in single culture when compared to that of *B. thermophilum* 20210 in co-culture with *L. delbrueckii* ssp. *bulgaricus* 2501 (Figure 5.2).

Figure 5.3 shows the log viable counts for *B. thermophilum* 20210 in single culture, *B. thermophilum* 20210 and *L. delbrueckii* ssp. *bulgaricus* 2515 in co-culture during a 24 hour incubation in MRS broth at 37°C. The relationship observed between *B. thermophilum* 20210 and *L. delbrueckii* ssp. *bulgaricus* 2515 was slightly different to that of *B. thermophilum* 20210 and *L. delbrueckii* ssp. *bulgaricus* 2501. According to Figure 5.3, the relationship between *B. thermophilum* 20210 and *L. delbrueckii* ssp. *bulgaricus* 2515 was an inhibitory one rather than an associative one. *L. delbrueckii* ssp. *bulgaricus* was largely inhibitory to the growth of *B. thermophilum* 20210, where there was only a 10% association between the two bacterial species. At 4 hours, the antagonism observed between the 2 bacterial organisms increased to 39% then dropped to 19% at 8 hours, then dropped significantly ($P<0.001$) at 12 hours, resulting in an inhibitory relationship (Figure 5.7).

This inhibitory effect exerted by *L. delbrueckii* ssp. *bulgaricus* 2515 could be attributed to its highly proteolytic nature. However, Shihata and Shah (2000) studied both strains and found that *B. thermophilum* 20210 is as proteolytic as *L. delbrueckii* ssp. *bulgaricus* 2515 when cultured alone in milk. The difference between both organisms is their rate of growth, where it is known that *B. thermophilum* 20210 is a slower growing organism than *L. delbrueckii* ssp. *bulgaricus* 2515, thus *L. delbrueckii* ssp. *bulgaricus* 2515 is able to out-compete *B. thermophilum* 20210 for essential nutrients and growth factors available in the medium.

Figure 5.4 represents the log viable counts for *L. acidophilus* 2405 in single culture, *L. acidophilus* 2405 and *S. thermophilus* 2002 in co-culture during a 24 hour incubation period in MRS broth at 37°C. *L. acidophilus* 2405 survived better when in association

with *S. thermophilus* 2002, especially at 0, 4, 8, and 12 hours. The viable counts for *L. acidophilus* 2405 were higher when co-cultured with *S. thermophilus* 2002 than when cultured alone (Figure 5.4). *L. acidophilus* 2405 was in turn inhibitory to the viability of *S. thermophilus* 2002, in particular, at 0, 12, and 24 hours which may be due to the effect the acid produced by *L. acidophilus* has on *S. thermophilus*.

Figure 5.5 represents the log viable counts for *L. acidophilus* 2405 in single culture, *L. acidophilus* 2405 and *L. delbrueckii* ssp. *bulgaricus* 2501 in co-culture during a 24 hour incubation period in MRS broth at 37°C. *L. acidophilus* significantly survived better when grown alone than when co-cultured with *L. delbrueckii* ssp. *bulgaricus* 2501 ($P<0.001$). *L. delbrueckii* ssp. *bulgaricus* was shown to be inhibitory to the survival of *L. acidophilus* 2405, where it was observed that only 28% association occurred between the two bacterial organisms (Figure 5.8). Lastly, Figure 5.6 represents the viable log counts for *L. acidophilus* 2405 in single culture, *L. acidophilus* 2405, and *L. delbrueckii* ssp. *bulgaricus* 2515 in co-culture during a 24 hour incubation period in MRS broth at 37°C. There was no particular trend observed between this combination, however, it can be noted that *L. acidophilus* 2405 appeared to survive better in single culture rather than co-cultured with *L. delbrueckii* ssp. *bulgaricus* 2515. This was significant at 24 h ($P<0.001$) where there was a 2-log difference between the growth of *L. acidophilus* 2405 alone when compared to that in co-culture. Therefore, it can be said that *L. delbrueckii* ssp. *bulgaricus* 2515 had an inhibitory effect on the survival of *L. acidophilus* 2405.

These findings contradict the earlier findings of the screening studies where it was observed that *L. acidophilus* 2405 inhibited the growth of *L. delbrueckii* ssp. *bulgaricus* 2501 and *L. delbrueckii* ssp. *bulgaricus* 2515 in the spot on lawn method (Table 5.1). These observations agree with that of Vincent *et al.* (1959), where no antibacterial activity was found when liquid cultures of *L. acidophilus* were tested. They reported that it was necessary to grow *L. acidophilus* on solid agar media for the substance(s) to be produced. Furthermore, extended incubation of agar media inoculated with the lactobacilli was necessary before appreciable inhibitory activity was detected. Other studies (Hamdan *et al.*, 1974; Vakil & Shahani, 1965) have shown that *L. acidophilus*

does produce inhibitory material in liquid media. However, in both these reports the inhibitory material was extracted from 48-hour milk cultures with organic solvents; no evidence was presented to indicate that sufficient activity was present in younger cultures to exert inhibitory action towards other bacteria.

5.4 Conclusions

In order to produce therapeutic benefits, the required minimum level of probiotic bacteria is claimed to be ~ 10^6 cfu/g of a product; however, recent surveys have shown poor viability of probiotic bacteria. This study screened for antagonism between yoghurt bacteria and probiotic bacteria which may be responsible for low viability of probiotic bacteria. A total of nine strains of yoghurt bacteria and probiotic bacteria were used in this study. *S. thermophilus* 2002 and both *L. delbrueckii* ssp. *bulgaricus* 2501 and 2515 strains showed strong inhibition which could be due to bacteriocin like inhibitory substance (BLIS) produced by both *L. acidophilus* strains. *Bifidobacterium* ssp. was not inhibited in this study.

Table 5.1 **Screening of yoghurt and probiotic bacteria for zones of inhibition.**

Producer Organism	Target Organism (Zone of inhibition mm)								
	S. thermophilus		*L. delbrueckii* ssp. *bulgaricus*		*L. acidophilus*		*Bifidobacterium* ssp.		
	2002	2014	2501	2515	2405	2415	20210	1941	20099
Control	-	-	-	-	-	-	-	-	-
ST 2002	-	-	-	-	-	-	-	-	-
ST 2014	-	-	-	-	-	-	-	-	-
LB 2501	-	-	-	-	-	-	-	-	-
LB 2515	-	-	-	-	-	-	-	-	-
LA 2405	+ 8 mm	-	+ 2 mm	+ 8 mm	-	+ 2 mm	-	-	-
LA 2415	-	-	+ 1 mm	-	-	-	-	-	-
BB 20210	-	-	-	-	-	-	-	-	-
BB 1941	-	-	-	-	-	-	-	-	-
BB 20099	-	-	-	-	-	-	-	-	-

Notes:
- = no inhibition
+ = inhibition observed

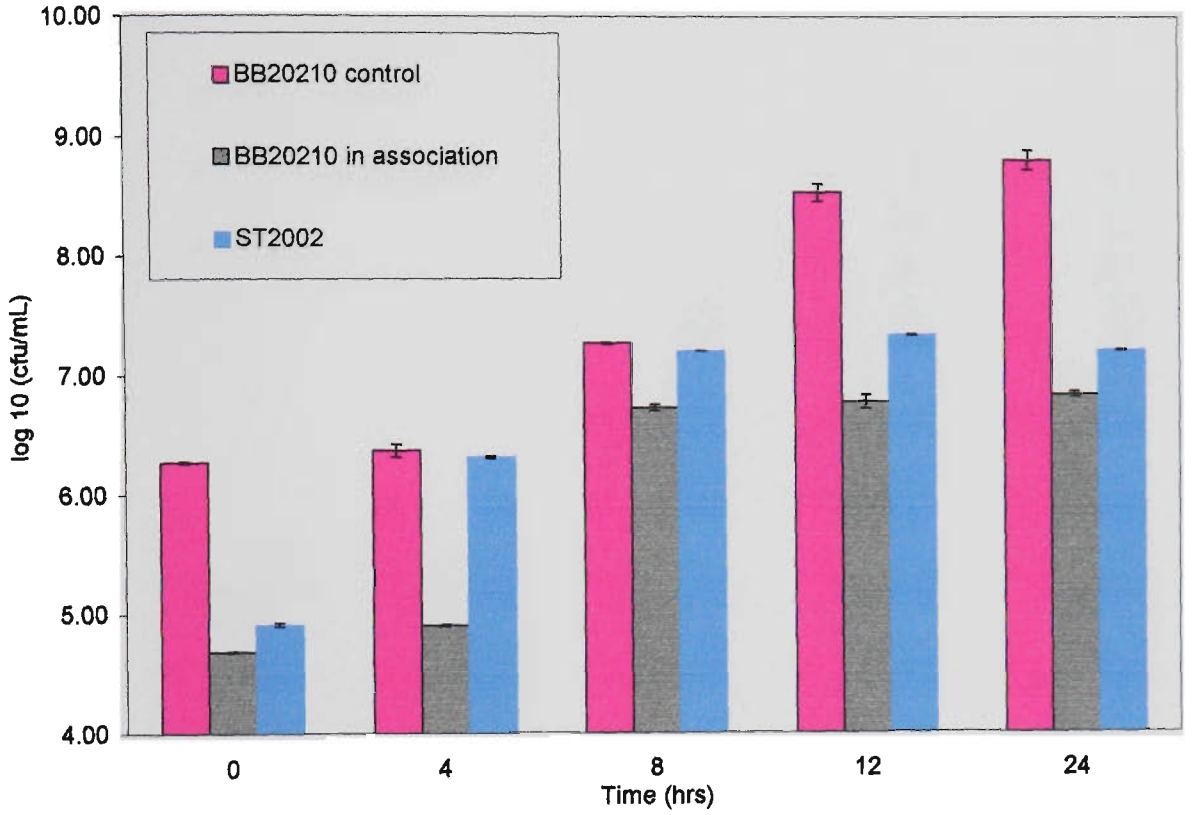

Figure 5.1 **Viable counts for *B. thermophilum* 20210 in single culture and in co-culture with *S. thermophilus* 2002 when grown in RSM at 37°C for 24 hours.**

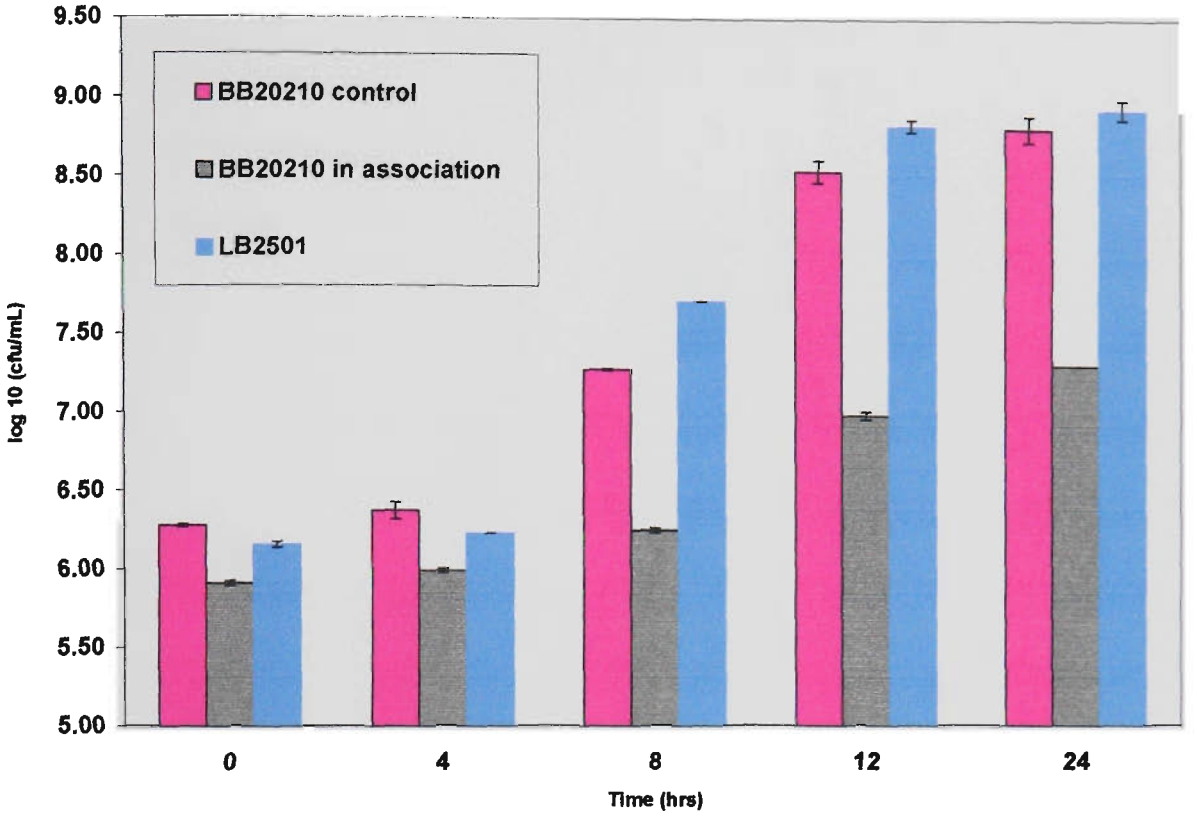

Figure 5.2 Viable counts for *B. thermophilum* 20210 in single culture and in co-culture with *L. delbrueckii* ssp. *bulgaricus 2501* when grown in RSM at 37°C for 24 hours.

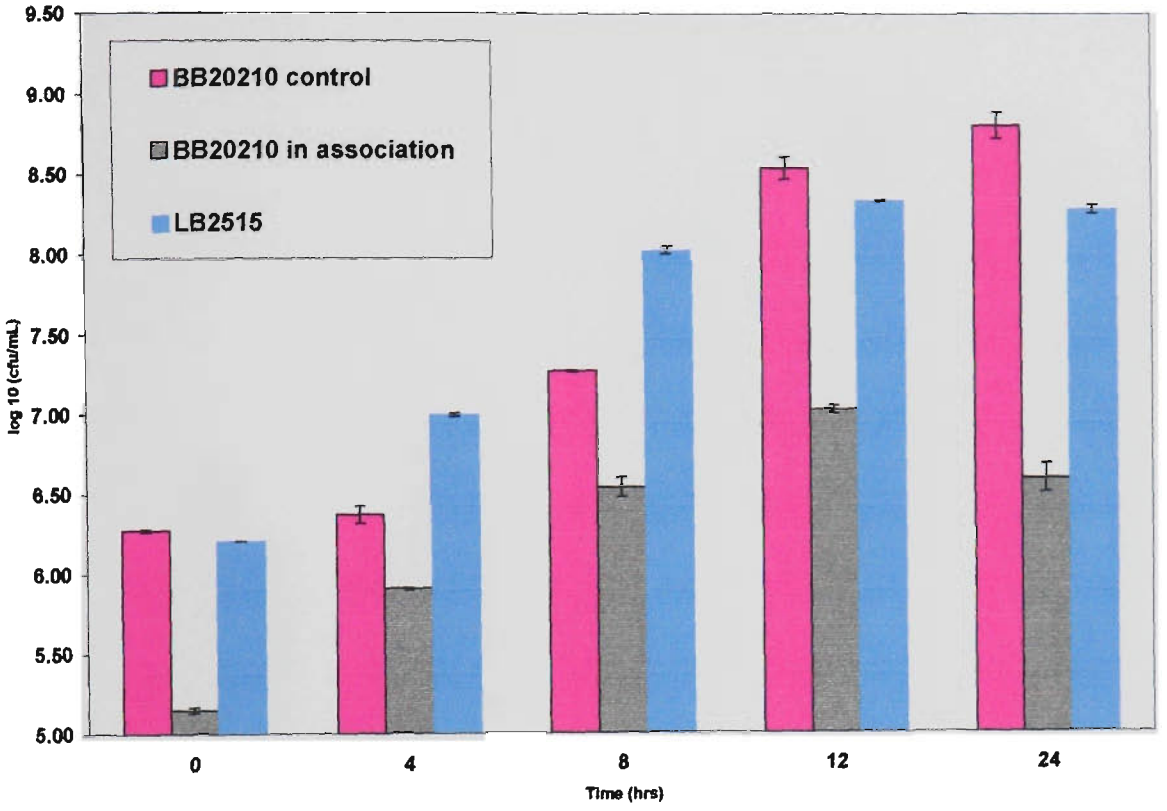

Figure 5.3 **Viable counts for *B. thermophilum* 20210 in single culture and in co-culture with *L. delbrueckii* ssp. *bulgaricus 2515* when grown in RSM at 37°C for 24 hours.**

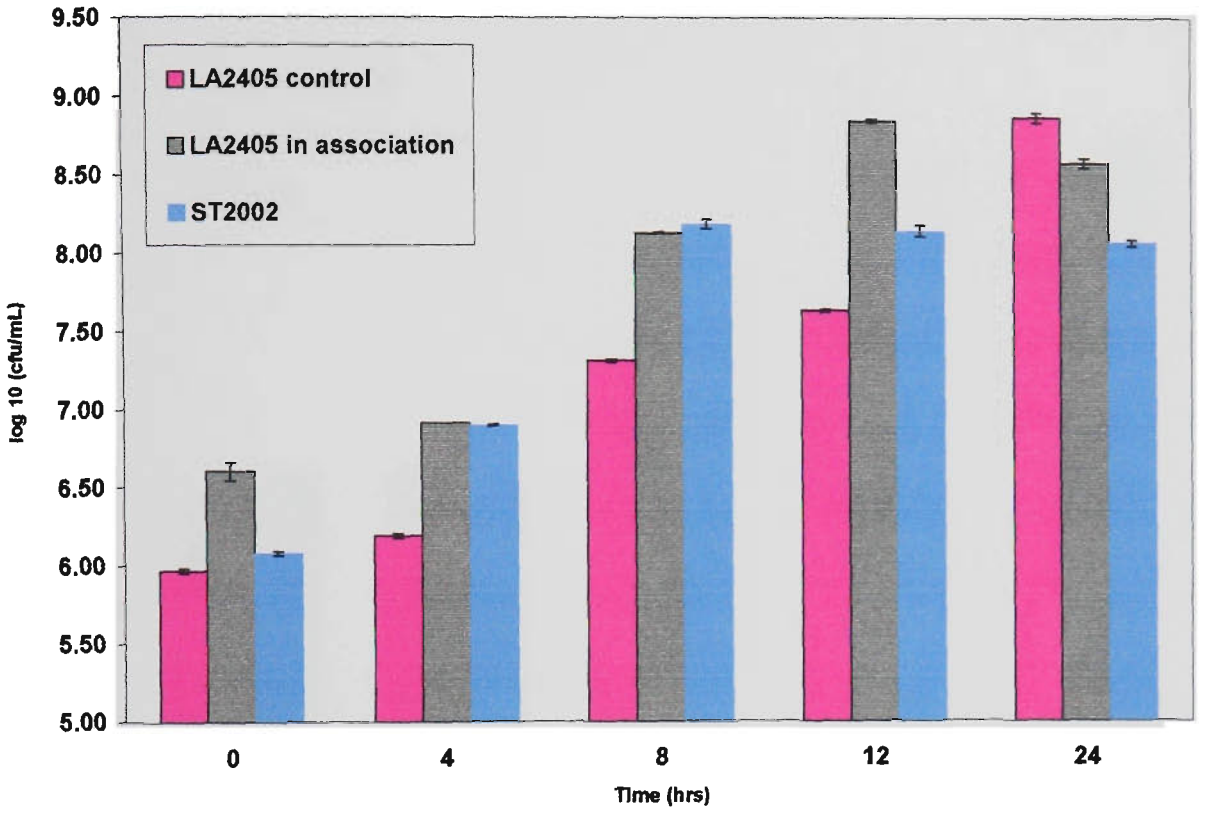

Figure 5.4 Viable counts for *L. acidophilus* 2405 in single culture and in co-culture with *S. thermophilus* 2002 when grown in RSM at 37°C for 24 hours.

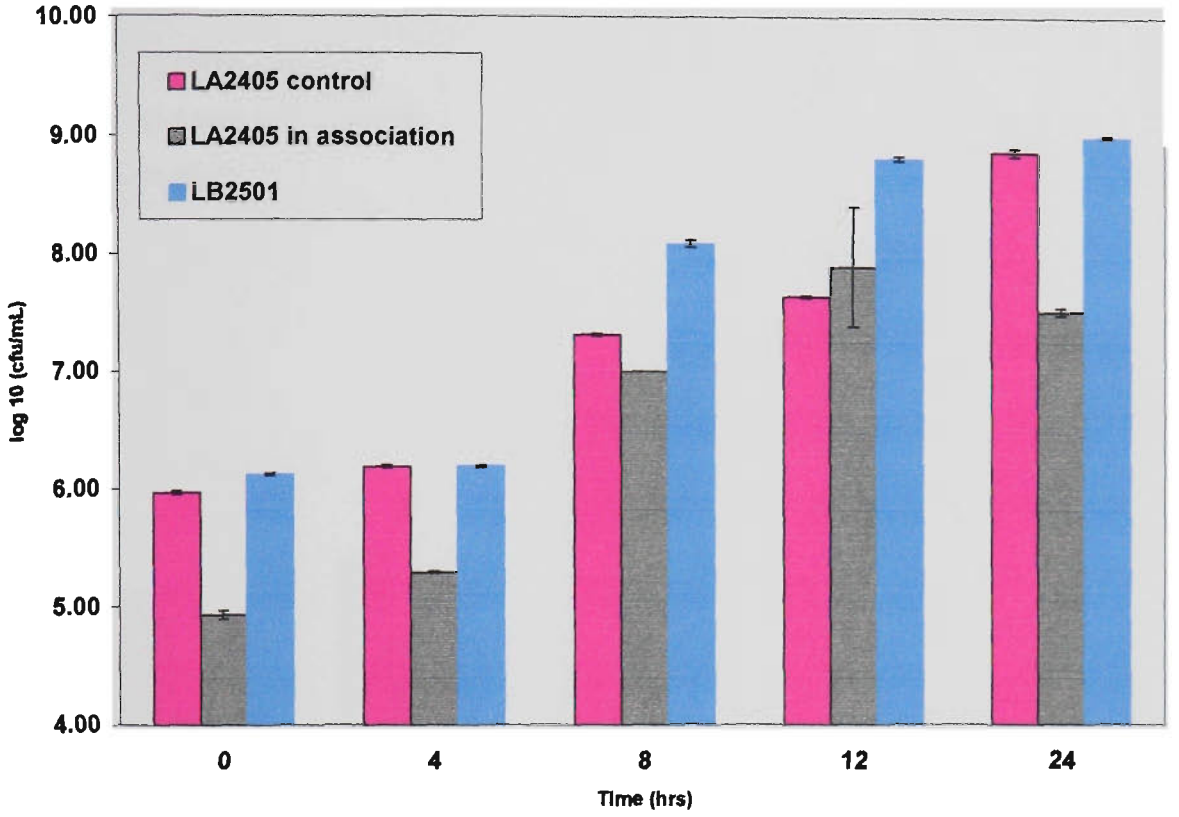

Figure 5.5 Viable counts for *L. acidophilus* 2045 in single culture and in co-culture with *L. delbrueckii* ssp. *bulgaricus* 2501 when grown in RSM at 37°C for 24 hours.

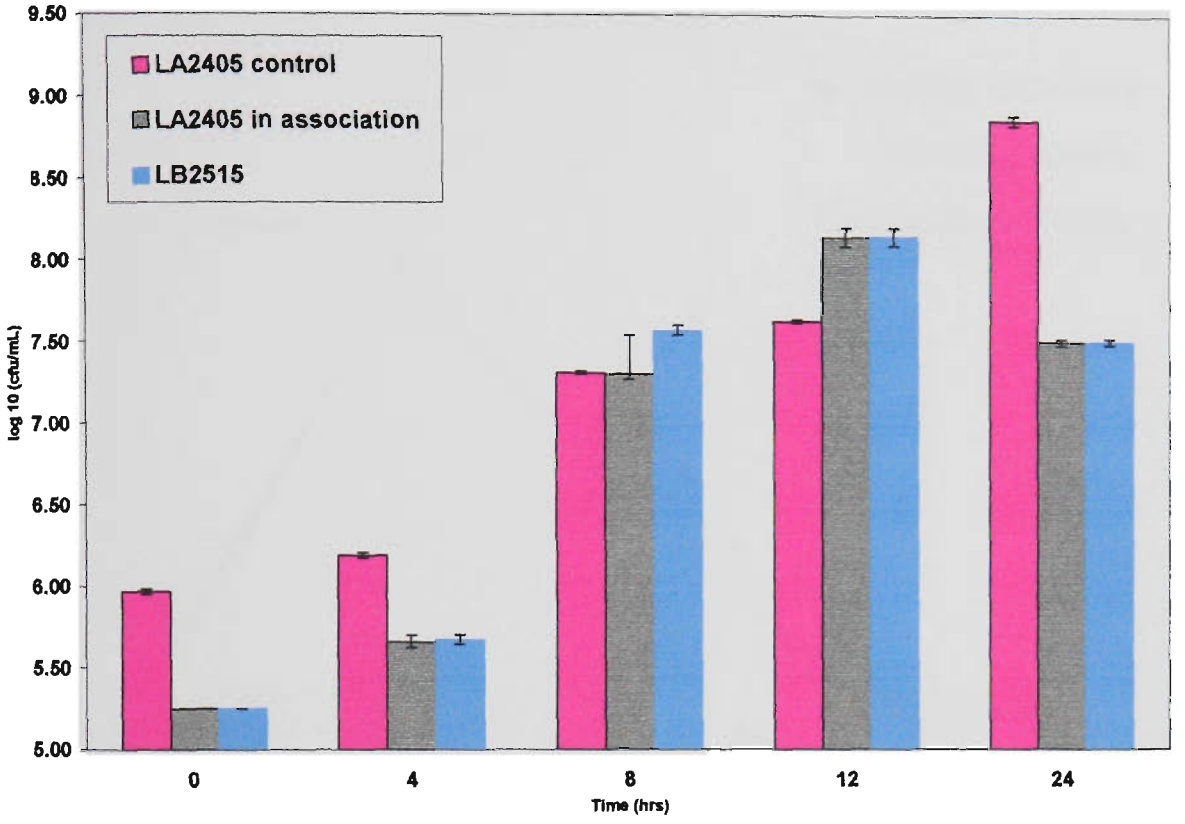

Figure 5.6 **Viable counts for *L. acidophilus* 2045 in single culture and in co-culture with *L. delbrueckii* ssp. *bulgaricus* 2515 when grown in RSM at 37°C for 24 hours.**

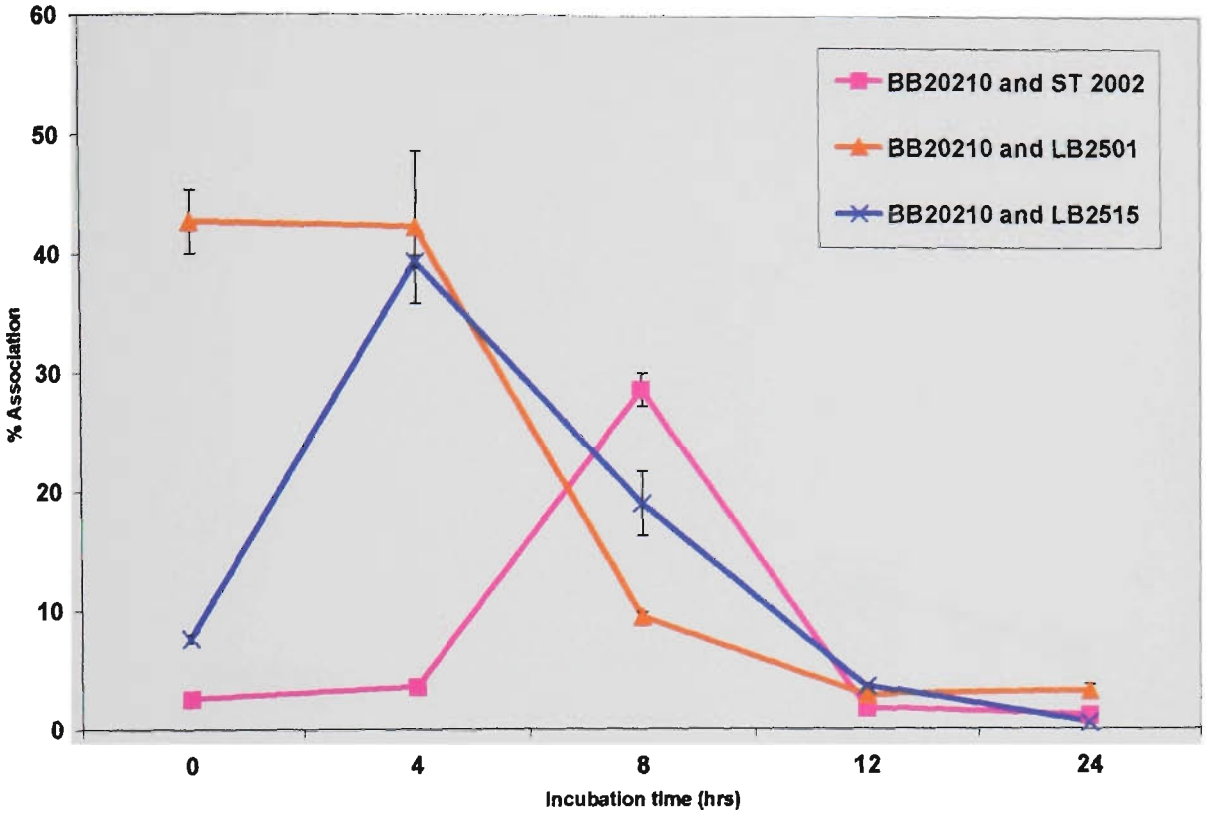

Figure 5.7 **Associative growth curves for *B. thermophilum* 20210 when co-cultured with *S. thermophilus* 2002, *L. delbrueckii* ssp. *bulgaricus* 2501 and *L. delbrueckii* ssp. *bulgaricus* in RSM at 37°C for 24 hours.**

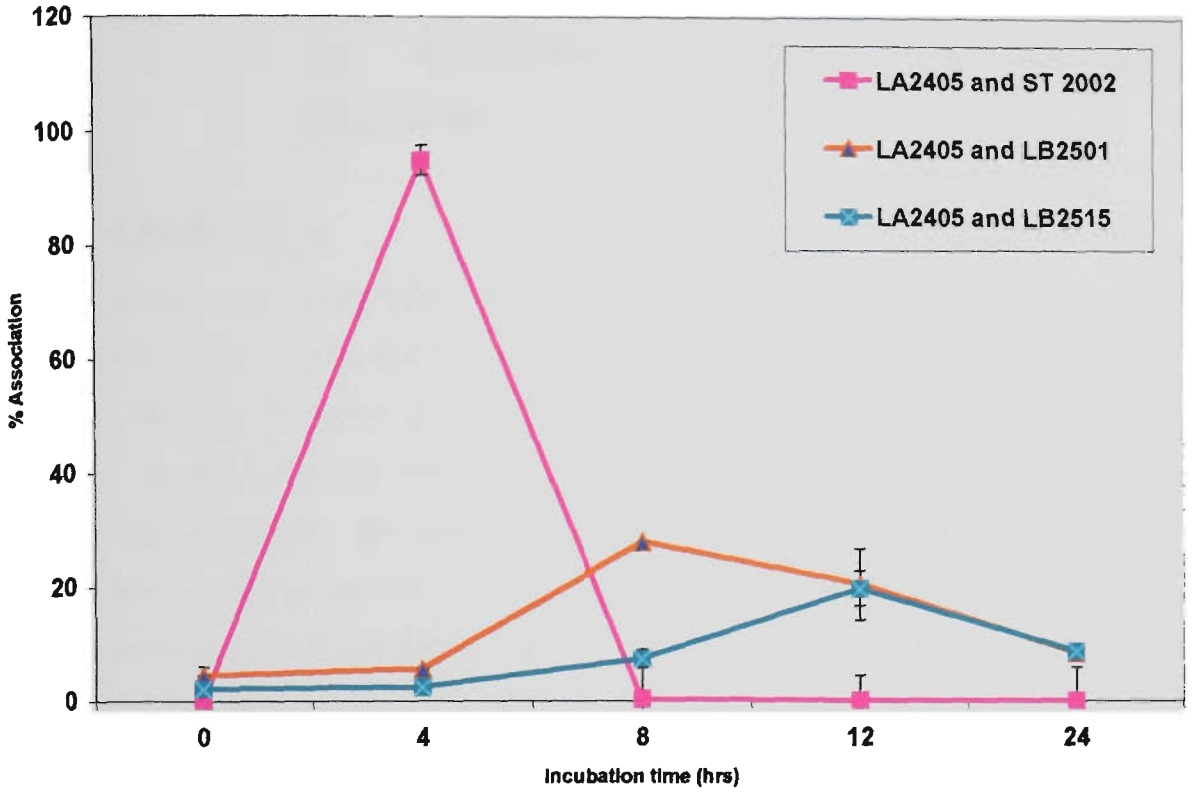

Figure 5.8 Associative growth curves for *L. acidophilus* 2045 when co-cultured with *S. thermophilus* 2002, *L. delbrueckii* ssp. *bulgaricus* 2501 and *L. delbrueckii* ssp. *bulgaricus* in RSM at 37°C for 24 hours.

Chapter 6 Degradation of casein and whey proteins by selected yoghurt and probiotic bacteria

6.1 Introduction

Growth of yoghurt and probiotic bacteria in milk depends on their ability to metabolize lactose (45-50 g/l), and hydrolyze caseins which are considered to be the main nitrogen source. Almost all caseins in milk are organized in casein micelles with an average diameter of about 120 nm containing 93% proteins and 7% inorganic salts, mainly calcium and phosphate. Bovine milk typically consists of 3.0-3.5% (w:w) protein, of which 80% is caseins that are defined chemically as the milk proteins that precipitate from solution at pH 4.6 and 20°C. The bovine casein group consists of α_{s1}-, α_{s2}-, β- and κ-casein, abbreviated as α_{s1}-CN, α_{s2}-CN, β-CN, and κ-CN, respectively, and they occur in the approximate proportions of 4:1:4:1 (w:w:w:w). The whey proteins, which account for ~20% of total milk protein are soluble at pH 4.6 and represent an excellent source of both functional and nutritious proteins. The main whey protein constituents are β-lactoglobulin (β-LG) and α-lactalbumin (α-LA) in a ratio of 3:1 (w: w). Immunoglobulins (Ig) and bovine serum albumin (BSA) are also considered to be whey proteins. Minor whey protein components include the immunoglobulins (Igs), glycomacropeptide, serum albumin, lactoferrin, proteose-peptones and numerous enzymes (Bobe *et al.*, 1998; Léonil *et al.*, 2000).

The importance of breakdown of casein into water soluble compounds with the action of proteolytic enzymes by microorganisms is now well recognized. LAB, which are used as starters for the preparation of dairy products, are known to be caseinolytic, although the extent of degradation varies with different strains.

Milk proteins are probably the best characterized of all food proteins. However, the existence of genetic and non-genetic polymorphism, as well as the application of technological treatments complicate their quantitative determination. Modifications such

as heat denaturation or proteolysis, common in the manufacture of many dairy products, give rise to complex, insoluble, new compounds and smaller peptides and amino acids whose analysis is not easily performed. In addition, information on the occurrence and amount of a particular protein or derived compound is extremely useful in the assessment of processing and adulterations. Analysis of milk proteins has been carried out using classical gel electrophoretic methods, isoelectric focusing and ion-exchange, hydrophobic interaction or reversed-phase HPLC among others (Recio *et al.*, 1997).

Capillary electrophoresis (CE) is a relatively new, fast-growing analytical technique that allows on-column detection of proteins and peptides at various wavelengths. It takes less time and manpower, requires smaller sample volumes than gel electrophoresis while resolution can be obtained. However, one major drawback of using CE for protein analysis is the adsorption of proteins onto the capillary wall, mainly due to interactions between negatively charged silanol groups of the silica surface of the capillary and positively charged protein regions. To reduce these undesirable interactions, coated capillaries are often used together with polymeric additives in the separation buffer (De Block *et al.*, 1998).

In recent years, CE has become an analytical technique with many applications in the study of dairy proteins and peptides. Caseins and casein lysates were studied by Kristiansen *et al.* (1994), Recio *et al.* (1997) and Otte *et al.* (1997). Whey proteins have been separated quantitatively by Otte *et al.* (1994) using untreated fused-silica capillaries banded with a layer of crosslinked polyacrylamide (De Block *et al.*, 1998).

CE is performed in a narrow fused-silica capillary (20-75 μm inner diameter) with an outer coating of polyamide to protect the glass fibre during handling. A short section of the polyimide coating is removed, usually by burning, to form a window for direct detection through the capillary wall. Analytes are continuously monitored by an on-capillary detector, usually UV/VIS. This makes the technique fully automatable with the possibility of running several samples in sequence. UV absorbency also allows the detection of small molecules like peptides and amino acids which are impossible to

visualize in polyacrylamide gel electrophoresis (PAGE) because of problems with fixing and staining. Furthermore, UV detection enables quantitative analysis in CE with an accuracy exceeding that possible in PAGE. CE resolves molecules, based on charge/mass ratio, in a buffer-filled fused-silica capillary under the influence of an electric field. The use of high electrical fields results in short analysis times and high efficiency and resolution.

The qualitative and quantitative analysis of proteins or protein digests by different high-performance liquid chromatography (HPLC) modes has become routine in most laboratories dealing with protein research and food analysis. There is no doubt that the present high-resolution chromatographic techniques offer many advantages for the separation and analysis of proteins. However, it has been recognized that the chromatography of proteins is in many respects different from that of small, more "rigid" biomolecules such as saccharides and organic acids. Modern laboratory practice requires not only a complete resolution of all compounds in a mixture in a short time but also, especially on a preparative scale, the highest possible recovery of sensitivity and resolution of modern HPLC systems.

Unless whole milk is to be analyzed, sample preparation usually includes the separation of the casein fraction from the whey fraction. This can be achieved during processing of the food, e.g., during the production of caseinates, whey protein isolates (WPIs), or whey protein concentrates (WPCs), or it has to be done prior to analysis. Possible means are centrifugation of the skim milk in the presence of Ca2+ to precipitate the casein fraction, renneting of the milk to obtain sweet whey, acidification of the milk to pH 4.6, with acid or lactic acid bacteria, to obtain acid whey. Acidification to pH 4.6 can also be used to remove denatured whey proteins. Other sample pretreatments can include the removal of low-molecular-weight compounds, such as salts and lactose, by dialysis, ultracentrifugation, and the addition of salts, e.g., $(NH_4)_2SO_4$, to remove aggregated, denatured proteins.

Quantification has been done separately for casein and whey proteins by using independent runs of gel electrophoresis, liquid chromatography, and capillary electrophoresis. Simultaneous separation and quantification of the casein and whey proteins has been reported by capillary zone electrophoresis, isoelectric focusing, and high-performance liquid chromatography (HPLC) (Bobe *et al.*, 1998).

The present chapter deals with the degradation of casein and whey fractions by selected yoghurt and probiotic bacteria. This study describes the detection of proteolytic activities in cell-free and cell wall extracts from various strains of yoghurt bacteria (*S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus*) and probiotic bacteria (*L. acidophilus* and *Bifidobacterium* ssp.). Proteolysis of whole bovine casein was used as a model system for this study. Polyacrylamide gel electrophoresis was used in this first part of the study to estimate the amount of degradation of milk proteins exhibited by the various bacterial strains tested. The second part of the study was based on the identification and quantification of proteolytic breakdown products released in a milk culture medium by selected yoghurt and probiotic bacterial strains. The method intended for this part of the study was CE but problems were encountered with optimization of the method chosen for use so reverse HPLC was used instead.

6.2 Materials and methods

6.2.1 SDS-PAGE electrophoresis

Cultures of *S. thermophilus* (2002 and 2014), *L. delbrueckii* ssp. *bulgaricus* (2501 and 2515), *L. acidophilus* (2405 and 2415), and *Bifidobacterium* ssp. (1912 and 20210) were selected for this study. The bacterial cultures were maintained and propagated according to the method described in section 3.2.1 in chapter 3. Intracellular and cell wall extracts of these bacterial cultures were prepared according to the method described in section 3.2.4.1 in chapter 3. Samples for SDS-PAGE electrophoresis were then prepared according to the method described in section 3.2.6.1 in chapter 3. The development of the SDS gels and analysis is described in section 3.2.6.1, 3.2.6.2, and 3.2.6.3 in chapter 3 respectively.

6.2.2 Capillary electrophoresis

Samples were prepared according to section 3.2.7.1 in Chapter 3. Electrophoresis buffers used were prepared according to section 3.2.7.2 in Chapter 3. Samples were then analysed by the method of capillary electrophoresis as described in section 3.2.7.3 in Chapter 3.

6.2.3 Reverse-phase high performance liquid chromatography (RP-HPLC)

The bacterial cultures used in this study are outlined in section 3.2.8.1 in Chapter 3. They were maintained and propagated according to section 3.2.1 in Chapter 3. Bacterial samples were then prepared according to the method described in section 3.2.8.2 in Chapter 3. The milk proteins in these bacterial samples were then identified and quantified by RP-HPLC by using the method described in section 3.2.8.3 in Chapter 3.

6.3 Results and Discussion

6.3.1 SDS-PAGE electrophoresis

Milk proteins (caseins and whey proteins) are the main nitrogen source for LAB, which utilize them with the action of exocellular proteinases, membrane-bound aminopeptidases and intracellular exopeptidases and proteinases. Thus the importance of breakdown of casein into water soluble compounds with the action of proteolytic enzymes by microorganisms is now well recognized. LAB are known to be caseinolytic, although the extent of degradation varies with different strains. Casein degradation was evaluated by SDS-PAGE electrophoresis at different incubation times with bacterial suspensions obtained from cultures grown in skim milk at 37°C.

Figure 6.1, 6.2, 6.3, 6.4, 6.5, 6.6, 6.7, 6.8, and 6.9 represent coomassie blue stains of SDS-PAGE gels of cell wall and intracellular samples collected from *S. thermophilus* 2002, *S. thermophilus* 2014, *L. delbrueckii* ssp. *bulgaricus* 2501, *L. delbrueckii* ssp. *bulgaricus* 2515, *L. acidophilus* 2401, *L. acidophilus* 2405, *L. acidophilus* 2415, *B. infantis* 1912, *and B. thermophilum* 20210, respectively. Samples collected at 0, 2, 4, and 6 hours were run by SDS-PAGE electrophoresis. Percentage hydrolysis of casein into the

major bovine milk proteins (α_s-casein, β-casein, and κ-casein) by the cell wall and intracellular extracts were analyzed and quantified in the samples obtained after 2, 4, and 6 hours of incubation.

Percentage amounts of α-casein, β-casein, and κ-casein utilised by the tested bacterial strains are presented in Table 6.1. According to the electrophoretic patterns and percentage hydrolysis results, the casein incubated with the intracellular samples were digested at a faster rate than that in cell wall extracts. Analysis of protein bands in the SDS-PAGE bands via density (Table 6.1) provided better quantification of degradation products than that estimated by visual observation of the gels. This was done by capturing an image of the gels via a Luminescent Image Analyser (LAS-1000 plus, Fujifilm, Tokyo, Japan) and the data were analysed with Image Gauge Software.

The software provided with the Luminescent Image Analyser (LAS-1000 plus, Fujifilm, Tokyo, Japan) calculated the area of the protein bands. The amount of protein in the bacterial sample was subtracted from that of the background protein. The same was calculated for the standard protein in the SDS-PAGE gel. The percentage amount of each milk protein in the bands detected in the gels are calculated using the following formulae:

Bacterial sample protein amount/protein standard X 100%.

The amount of protein hydrolysed by each bacterial culture was calculated by subtracting the percentage amount of protein detected from 100%.

Table 6.1 represents the percentage amounts of α_s-casein, β-casein, and κ-casein products hydrolysed in cell wall and intracellular fractions of *S. thermophilus* 2002, *S. thermophilus* 2014, *L. delbrueckii* ssp. *bulgaricus* 2501, *L. delbrueckii* ssp. *bulgaricus* 2515, *L. acidophilus* 2401, *L. acidophilus* 2405, *L. acidophilus* 2415, *B. thermophilum* 20210, and *B. infantis* 1912 resulting from the degradation of casein. According to Table 6.1, most bacterial strains showed 0% hydrolysis of α_s-casein, β-casein, and κ-casein at the cell wall level when compared to that at the intracellular level. These findings agree

with that of Kalantzopoulos *et al.* (1990), where they had demonstrated that casein incubated with IE fractions of lactobacilli and streptococci strains was digested faster than when incubated with CWE. *S. thermophilus* 2002 completely degraded the protein κ-casein (100% hydrolysis at all hours), where 0% was detected in the SDS-PAGE. This was observed in both cell wall and cell-free fractions. At the intracellular level, *S. thermophilus* 2002 degraded α-casein and β-casein at a slower rate (when compared to κ-casein) where 25% and 30%, respectively, was detected after 2 hours of incubation.

S. thermophilus 2014 showed a different pattern to that of *S. thermophilus* 2002. *S. thermophilus* 2014 also attacked α-casein more readily at the CWE level when compared to 0% hydrolysis at the IE level, implying the presence of proteinase activity occurring at the cell wall level. *S. thermophilus* had a preference for α_s-casein degradation at the cell wall level showing 0% hydrolysis of the other milk proteins studied. *S. thermophilus* 2014 appeared to hydrolyse β- and κ-casein at the intracellular level agreeing with previous studies of Kalantzopoulos *et al.* (1990) (Table 6.1).

Both *L. delbrueckii* ssp. *bulgaricus* 2501 and 2515 readily attacked all casein fractions, particularly at the intracellular level. Most of the hydrolysis occurred after 4 hours of incubation. *L. delbrueckii* ssp. *bulgaricus* 2501 and 2515 did not show any signs of milk protein utilisation at the cell wall level where 0% of all milk proteins was utilised. Hydrolysis of κ-casein by *L. delbrueckii* ssp. *bulgaricus* 2501 and 2515 at the intracellular level increased with time of incubation (Table 6.1).

L. acidophilus 2405 had a preference for milk protein degradation at the intracellular level, where β-casein hydrolysis increased with incubation time, indicating that 29%, 35%, and 70% of the milk protein was used up at 2, 4, and 6 hours respectively. *L. acidophilus* utilised 36% and 30% of κ-casein at 4 and 6 hours respectively (Table 6.1). *L. acidophilus* 2415 appeared to degrade the casein fractions at the intracellular level only after 6 hours of incubation which would indicate that it is a slower grower than that of *L. acidophilus* 2405. *L. acidophilus* 2415 also appeared to degrade the casein fractions at

the cell wall level after 2 hours of incubation which would indicate that proteinase activity is present at that cellular level.

On the other hand, *B. thermophilum* 20210, and *B. infantis* 1912 did show caseinolytic activity, but a slightly different pattern to that observed with the other bacterial strains tested. *B. infantis* 1912 appeared to show casein hydrolysis at both cell levels, however slower than that of the other probiotic strain and yoghurt strains, which agrees with earlier findings in this study. *Bifidobacterium* ssp. are much slower growers in milk when compared with the other bacteria cultures (Shihata & Shah, 2000). *B. infantis* 1912 showed a preference for α_s-casein degradation where 100% was utilised at the end of the 6 hour incubation period. *B. infantis* 1912 also degraded β-casein at the cell wall level faster than that observed at the intracellular level. On the other hand, κ-casein was not readily utilised by *B. infantis* 1912, only utilizing 26% at the cell wall level after 4 hours of incubation (Table 6.1).

B. thermophilum 20210 appeared to hydrolyse all casein fractions at both cell levels (Table 6.1). These findings agree with those found earlier where *B. thermophilum* 20210 had previously showed significant proteolytic activity and aminopeptidase activity (Shihata & Shah, 2000). *B. thermophilum* 20210 is a much faster grower than that of *B. infantis* 1912 where hydrolysis of the casein fractions started as early as after 2 hours of incubation which means it is a more fastidious organism. *B. thermophilum* 20210 had a preference for milk protein degradation at the cell wall level when compared to that at the intracellular level. High levels of milk protein utilisation occurred at the cell wall level which indicates proteinase activity occurring.

Some of these study findings agree with earlier research where *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* individually degrade κ-casein more rapidly than α_s- or β-caseins. It has been reported that lactic cultures degrade α- or κ-casein more readily than β-fractions, whereas others have observed that β- and κ-casein fractions are easily hydrolysed as compared to α_s-fractions. Caseinolytic activity varies from research to

another where findings depending on the method of analysis adopted and bacterial strains tested. Results will vary within bacterial strains (Singh & Sharma, 1983).

6.3.2 *Capillary electrophoresis*

The method of capillary electrophoresis was not successful in the identification of protein degradation patterns exhibited by the yoghurt and probiotic strains in this study. Different methods were adopted with little success of a separation and quantification of milk proteins. Only one separation was achieved but the clarity of the separation was not optimized and could not be repeated more than twice without equipment failure. One possible reason attributed to the failure of this method could be due to the crystallization of the urea present in the running buffer used in the system. Fresh buffer was made each time before each run; however separation was not successful. After many attempts, new methods, different capillaries, it was decided that reverse-phase HPLC was to be adopted as the preferred method.

6.3.3 *Reverse-phase high performance liquid chromatography (RP-HPLC)*

The RP-HPLC method used was that developed by Bobe *et al.* (1998) where separation and quantification of the six major bovine milk proteins was possible in the same run. The bovine casein group (α_s, β, and κ-casein) and the whey proteins (β-lactoglobulin and α-lactalbumin) were separated and quantified using reverse-phase HPLC. Bovine milk proteins were quantitatively determined by using standard curves which were developed by measuring peak areas at various known amounts of injected milk proteins. The standard curves were used to calculate the amount of protein represented by peak areas from bacterial samples of unknown composition. A control was also used where dairy farmer milk (which was used throughout this study) was analysed at 0 hour (Table 6.2). This was used to compare to the milk samples that were inoculated with the bacterial preparations. Samples were taken at 4, 6 and 12 hours of incubation. The usual fermentation time for yoghurt making preferred by yoghurt manufacturers is 6 hours, however, in this study, milk was incubated till 12 hours. This is because most data available from studies done in proteolysis has been carried out for several days and not

just hours, so incubation periods were extended so that some comparisons can be made to other research studies done.

Amount of α_s-casein (mg/mL) quantified in single cultures of *S. thermophilus* 2014, *S. thermophilus* 2002, *L. delbrueckii* ssp. *bulgaricus* 2501, *L. delbrueckii* ssp. *bulgaricus* 2515, *L. acidophilus* 2415, *L. acidophilus* 2405, *B. thermophilum* 20210, *and B. longum* 1941, and *B. pseudolongum* 20099 are represented in Figure 6.10. Figure 6.11 shows the amount of α_s-casein (mg/mL) quantified in mixed cultures of *S. thermophilus* 2002 + *L. delbrueckii* ssp. *bulgaricus* 2501, *S. thermophilus* 2014 + *L. delbrueckii* ssp. *bulgaricus* 2515, *S. thermophilus* 2014 + mix, *S. thermophilus* 2002 + mix, *L. acidophilus* 2415 + *B. thermophilum* 20210, and *L. acidophilus* 2405 + *B. longum* 1941. The amounts of α_s-casein (mg/mL) quantified in starter culture strains *S. thermophilus* T-1 (T-1 ST), S. *thermophilus* Y-1 (Y-1 ST), S. *thermophilus* T-4 (T-4 ST), *L. delbrueckii* ssp. *bulgaricus* Y-2 (Y-2 LB), *L. acidophilus* T-1 (T-1 LA), *Bifidobacterium* ssp. T-1 (T-1 BB), and *Bifidobacterium* ssp. isolated from BB-12 culture are shown in Figure 6.12.

According to Figure 6.10, *S. thermophilus* 2014 and *L. acidophilus* 2405 showed a preference for α_s-casein degradation when compared to the other bacterial single cultures studied. The amount of α_s-casein quantified in *S. thermophilus* 2014 and *L. acidophilus* 2405 at 12 hours were 1.63 and 2.42 mg/mL, respectively, which was significantly lower ($P<0.010$) than the other single cultures studied at 12 hours. *S. thermophilus* 2002 also showed a preference for α_s-casein degradation at 12 hours, where 4.36 mg/mL was quantified. Peak sizes corresponding to α_s-casein increased in size when quantified in the other yoghurt bacterial strains studied, *L. delbrueckii* ssp. *bulgaricus* 2501 and 2515 when compared with *S. thermophilus* 2014 and *L. acidophilus* 2405. This was evident from Figure 6.10 which showed an increase in the band size corresponding to α_s-casein over the 12 hour incubation period. This may be due to hydrolysis of the α_s-casein and thus the subsequent covalent bonding of peptides to this protein leading to an increase in the size of the peak corresponding to the α_s-casein fraction.

On the other hand *Bifidobacterium* ssp. (1941 and 20099) appeared to hydrolyse α_s-casein at a slower rate which was found in a previous study (Shihata & Shah, 2000). They are slow growers and need a longer incubation time than that of 12 hour to be able to completely breakdown α_s-casein into smaller peptides. Peak sizes corresponding to α_s-casein increased in size which indicates that *Bifidobacterium* ssp. are actively breaking down the casein fraction and forming new bonds with smaller peptides, thus the increase in peak size. *B. thermophilum* (20210) appeared to break the α_s-casein fraction into a smaller peptide which indicates that it is actively degrading the casein fraction at a faster rate than the other *Bifidobacterium* ssp. strains studied, thus the smaller peak size (Figure 6.10).

When the mixed bacterial cultures were studied for α_s-casein hydrolysis, *S. thermophilus* 2002 + mix, *S. thermophilus* 2014 + mix, and *L. acidophilus* 2415 + *Bifidobacterium thermophilum* 20210, showed significant hydrolysis ($P<0.017$). This can be seen in Figure 6.11 where the peaks corresponding to α_s-casein quantified in these mixed cultures decreased in size over time when compared with other mixed cultures studied and the control milk at 0 hours. Figure 6.11 shows that α_s-casein peak size decreased during the 12 hour incubation period in the mixed cultures of *S. thermophilus* 2002 + mix, *S. thermophilus* 2014 + mix, and *L. acidophilus* 2415 + *Bifidobacterium thermophilum* 20210. At 12 hours, 0.07, 3.50 and 4.84 mg/mL of α_s-casein was quantified by each of *S. thermophilus* 2002 + mix, *S. thermophilus* 2014 + mix, and *L. acidophilus* 2415 + *Bifidobacterium thermophilum* 20210, respectively. It appears that the combination of yoghurt bacteria in a mixed culture did degrade α_s-casein at a faster rate than when cultured alone as would have been expected, indicating they are not inhibitory and are actively breaking down existing bonds and forming smaller peptide fractions. This was expected because previous studies have shown that *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus* have a symbiotic relationship, where the former organism produces amino nitrogen for the latter organism (Shihata & Shah, 2000; Dave & Shah, 1998).

Degradation patterns of α-casein exhibited by starter culture strains were found to be significantly different ($P<0.012$ at 12 hours) to that of single and mixed bacterial cultures,

discussed earlier. In Figure 6.12, it can be observed that there was not much change in α_s-casein degradation patterns during the 12 hour incubation period (42°C) by the starter culture strains studied. During the 12 hour incubation period, the amount of α_s-casein did not change over time which is evident by the corresponding α_s-casein peak size which indicates that α-casein hydrolysis was either happening at a slower rate when compared with the other cultures or needed more incubation time like that of *Bifidobacterium* strains. These findings agree with earlier studies discussed in Chapter 4 and those of Shihata & Shah (2000) where it was shown that the same starter culture strains studied here demonstrated weak proteolytic activity when compared with the other bacterial cultures studied. This indicates that these starter culture strains are not as proteolytic as the other bacteria studied.

A similar trend to that of α_s-casein hydrolysis was observed with β-casein degradation patterns. Degradation patterns for β-casein in single yoghurt and probiotic cultures, mixed yoghurt and mixed probiotic cultures, and starter culture strains are represented in Figures 6.13, 6.14, and 6.15, respectively. When β-casein hydrolysis was studied in *L. acidophilus* 2405, it appeared that the peak corresponding to β-casein decreased in size over time from 3.44 mg/mL at 4 hours to 0.73 mg/mL at 12 hours (Figure 6.13). This indicates that *L. acidophilus* 2405 is actively hydrolysing β-casein, breaking down existing bonds and forming new smaller peptides. Similar observations were also seen with the yoghurt bacteria, *S. thermophilus* 2002 and *S. thermophilus* 2014 (Figure 6.13).

The other yoghurt bacteria, *L. delbrueckii* ssp. *bulgaricus* 2501 and 2515 did not appear to degrade β-casein at the same rate as that exhibited by *S. thermophilus* strains. This is observed in Figure 6.13 where the amount of β-casein quantified did not change significantly ($P<0.006$) during the 12 hour incubation period, when compared to the lower peak sizes observed with *S. thermophilus* strains. This would suggest that *L. delbrueckii* ssp. *bulgaricus* cultures did not have a preference for β-casein utilization or were slightly slower at degrading β-casein when compared with *S. thermophilus* strains.

Degradation patterns for β-casein in the mixed yoghurt and probiotic bacteria cultures can be seen in Figure 6.14. When *S. thermophilus* 2002 was mixed with other bacterial cultures, β-casein was hydrolysed at a faster rate when compared with the other mixed cultures tested. This is indicated by the presence of a larger peptide which decreases in size at 12 hours (0.54 mg/mL). The peak corresponding to β-casein slightly increased in size at 6 hours producing 4.86 mg/mL, then, significantly decreased to 0.54 mg/mL at 12 hours ($P<0.028$) which indicates that β-casein has been degraded by the mixed culture, forming smaller oligopeptides. The same pattern of β-casein hydrolysis was also observed with the combination mix of *L. acidophilus* 2405 and *B. longum* 1941, where the peak size corresponding to β-casein increased at 6 hours then decreased at 12 hours. A possible explanation for this trend could be due to the bacterial cultures actively degrading the available milk proteins earlier on during their growth, thus breaking down the covalent bonds within the β-casein fraction and linking covalent bonds with other peptides available leading to the increased band size.

In the other yoghurt mix, *S. thermophilus* 2002 + *L. delbrueckii* ssp. *bulgaricus* 2501, the peak corresponding to β-casein increased in size throughout the 12 hour incubation period. This could be explained in terms of the highly proteolytic nature of both of *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* and their symbiotic relationship, where the covalent bonds within the protein fraction are broken down to form new links with other peptides, thus forming a larger peptide fraction. This culture mix could be used to improve the viability of probiotic bacteria in a fermented milk product providing that this trend is also observed with other casein fractions and whey proteins. The peak corresponding to β-casein decreased in size significantly ($P<0.028$) between 6 and 12 hours in the combination mix of *L. acidophilus* 2415 and *B. thermophilum* 20210, indicating that they are actively utilising the β-casein fraction, forming new covalent bonds and forming new peptide fractions.

Significant ($P<0.034$) patterns of β-casein hydrolysis were observed with the starter culture strains as seen in Figure 6.15. Yoghurt starter culture strains, T-1 ST, Y-1 ST, T-4 ST, and Y-2 LB, appeared to be actively degrading the β-casein fraction over time,

where the peak corresponding to β-casein decreased in size during the 12 hour incubation period. Probiotic starter culture T-1 LA was also shown to be actively degrading β-casein where there was a significant ($P<0.034$) decrease in β-casein peak size between 6 and 12 hours (Figure 6.15). This indicates that the β-casein fraction is breaking down to form new and smaller peptide fractions. However, BB-12 did not appear to show much β-casein hydrolysis, indicated by the small variation in peak size over the 12 hour incubation period. This could be because *Bifidobacterium* ssp. is known to be a slow grower in milk and thus breaking down the β-casein at a much slower rate than other bacteria.

Figure 6.16 represents degradation patterns for κ-casein for single yoghurt and probiotic cultures. The peak size corresponding to the κ-casein fraction was shown to increase in size over the incubation time with both *S. thermophilus* 2002 and *L. delbrueckii* ssp. *bulgaricus* 2515. This would mean that these two yoghurt cultures are fast growing, breaking down milk proteins, in particular producing new and larger κ-casein fractions, with no apparent need for κ-casein utilisation at that stage of their bacterial growth. On the other hand, there was a significant decrease ($P<0.006$) in κ-casein peak size observed with *L. delbrueckii* ssp. *bulgaricus* 2501 which means that *L. delbrueckii* ssp. *bulgaricus* 2501 is breaking down dipeptide bonds within the κ-casein fraction of milk proteins and forming new and smaller peptides. This observation agrees with earlier studies of Shihata and Shah (2000) where *L. delbrueckii* ssp. *bulgaricus* 2501 was shown to be a slower grower than *L. delbrueckii* ssp. *bulgaricus* 2515. There was a significant decrease ($P<0.006$) in the peak size corresponding to κ-casein, where 4.22 mg/mL and 1.81 mg/mL was quantified at 4 and 6 hours, respectively, when *S. thermophilus* was incubated in milk for 12 hours (Figure 6.16).

Figure 6.17 shows that both *L. acidophilus* strains 2415 and 2405 were utilising the κ-casein available in the culture medium which indicates that they were being active. This means that peptide bonds are being broken down forming new and smaller peptide fractions (Webb et al., 1978). On the other hand, the other probiotic bacterial strains studied (*B. thermophilum* 20210, *B. longum* 1941, and *B. pseudolongum* 20099), did not

appear to be degrading κ-casein at the same rate as that as the other bacteria studied, indicating that they are a slow growing organism. The peak corresponding to κ-casein appeared to increase in size by the twelfth hour of incubation indicating that the κ-casein fraction was increasing in size forming new dipeptide bonds with other peptides forming a much larger oligopeptides.

Degradation patterns of κ-casein by mixed cultures are represented in Figure 6.17. It is observed that all bacterial cultures studied degraded κ-casein to an extent when compared to that of κ-casein in the control milk (7.18 mg/ml), however, this was not significantly different ($P<0.456$). Mixed cultures of *S. thermophilus* 2014 + *L. delbrueckii* ssp. *bulgaricus* 2515 and *S. thermophilus* 2002 + mix showed a decrease in the size of the peak corresponding to the κ-casein fraction indicating that they are actively hydrolysing κ-casein and breaking it down into smaller peptide fractions. These particular combinations consist of bacterial organisms that are proteolytic in nature and thus would be in competition for available nutrients. Other mixed bacterial cultures studied showed an increase in the peak size corresponding to κ-casein which means they are actively breaking down available milk proteins at a slower rate, where some peptides may bind with this protein fraction leading to an increase in the peak size (Figure 6.17).

Figure 6.18 shows quantitation of κ-casein in starter culture bacterial strains studied during a 12 hour incubation period. When κ-casein content produced by the starter culture strains was compared to that of the control milk, it was observed that there was not much change in the peak size corresponding to the κ-casein fraction, which indicates that during their earlier stages of growth, not much κ-casein hydrolysis is occurring. This could be due to the fact that they are less proteolytic than the other bacterial cultures tested and thus are slower at breaking down milk proteins and thus utilising them.

Quantification of α-lactalbumin from single yoghurt and probiotic cultures are represented as mg/mL in Figure 6.19. When compared to the control milk (Table 6.2), there appears to be a significant ($P<0.018$) increase in the peak size corresponding to the α-lactalbumin protein fraction during the first 4 hours observed with the yoghurt cultures

of *S. thermophilus* 2014, *S. thermophilus* 2002, *L. delbrueckii* ssp. *bulgaricus* 2515, and the probiotic culture of *L. acidophilus* 2415. This was followed by a significant decrease at 12 hours ($P<0.018$). This was observed with the yoghurt cultures of *S. thermophilus* 2014 and *L. delbrueckii* ssp. *bulgaricus* 2515, and the probiotic cultures of *L. acidophilus* 2405 and *B. pseudolongum* 20099. On the other hand, during the 12 hour incubation period, there was an increase in the peak size corresponding to the α-lactalbumin fraction observed with the other *Bifidobacterium* ssp. studied and *L. delbrueckii* ssp. *bulgaricus* 2501 (Figure 6.19). The heights of the α-lactalbumin peaks were reduced and several additional peaks appeared with migration times closer to those of α-lactalbumin. Previous research (Garcia-Risco *et al.*, 1999) has indicated that this could be due to the increased formation of polymers, involving casein polypeptides and whey proteins covalently linked or disulphide-linked through carbonyl intermediates.

Changes in α-lactalbumin content within mixed yoghurt and probiotic cultures are presented in Figure 6.20. *S. thermophilus* 2014 + mix and *S. thermophilus* 2002 + mix showed a slight increase in the peak size corresponding to the α-lactalbumin protein fraction when compared to that of the control during the first 4 hours of incubation (Table 6.2). This was then followed by a decrease in the peak size corresponding to the α-lactalbumin fraction between 4 and 6 hours observed for *S. thermophilus* 2014 + mix. According to Figure 6.20, there was no particular trend observed for α-lactalbumin utilisation by the mixed bacterial cultures. It mostly appeared that the mixed bacterial cultures are hydrolysing the α-lactalbumin protein forming new bonds with other peptides, however not significantly different ($P>0.576$), where the differences among the various combinations are not great enough to exclude the possibility that the difference is due to random sampling variability.

Figure 6.21 represents degradation patterns for α-lactalbumin shown by starter culture bacterial strains. It is observed that not much change in α-lactalbumin peak size had occurred during the first 4 hours of incubation indicating slow growth. However at 6 and 12 hours, the peak size of α-lactalbumin in the BB-12 culture increased to 0.79 mg/mL. This indicates that there is some activity occurring at this stage of bacterial growth. The

rest of the starter culture strains did not appear to be actively degrading or utilising α-lactalbumin. The differences in the mean concentration for α-lactalbumin among the starter culture strains are not great enough to exclude the possibility that the difference is due to random sampling. There isn't a statistically significant difference (P>0.063) among the various groups.

Figure 6.22 represents the amount of β-lactoglobulin A quantitated in single yoghurt and probiotic bacterial cultures. Compared to the control milk (Table 6.2), there appeared to be a slight decrease in β-lactoglobulin A peak size observed at 4 hours for all single bacterial cultures, although not statistically significant (P>0.061). There was also a slight decrease in β-lactoglobulin A peak size between 6 and 12 hours observed for all single bacterial cultures studied except for *S. thermophilus* 2014, *L. delbrueckii* ssp. *bulgaricus* 2515, and *B. longum* 1941. When yoghurt and probiotic bacteria were mixed together in various combinations, there was an increase in β-lactoglobulin A peak size at 4 hours observed in the combinations of *L. acidophilus* 2405 + *B. longum* 1941 and that of *S. thermophilus* 2014 + *L. delbrueckii* ssp. *bulgaricus* 2515 (Figure 6.23). No particular trends were seen with respect to degradation of β-lactoglobulin A as was exhibited by the bacterial combinations. There were no changes in β-lactoglobulin A peak size observed during the 12 hour incubation except for a slight decrease observed in the bacterial combinations of *L. acidophilus* 2405 + *B. longum* 1941 and that of *S. thermophilus* 2014 + *L. delbrueckii* ssp. *bulgaricus* 2515 (Figure 6.23), but not significant enough (P>0.480) to exclude the possibility that the difference is due to random sampling variability.

All starter culture bacterial strains studied showed a decrease in the peak size corresponding to the β-lactoglobulin A whey protein fraction during the first 4 hours of incubation (Figure 6.24). A sharp increase in β-lactoglobulin A peak size was observed with T-1 LA at 6 hours which indicates that this organism was actively breaking down the available milk proteins. On the other hand, β-lactoglobulin A peak size decreased in T-1 BB culture at 6 and 12 hours.

When single yoghurt and probiotic bacterial cultures were subjected to analysis for β-lactoglobulin B content, *L. delbrueckii* ssp. *bulgaricus* 2501 showed a significantly smaller peak corresponding to the β-lactoglobulin B fraction during the first 4 hours of incubation (Figure 6.25). This continued over the 12 hour period of incubation which indicates that this particular organism was actively utilising any available β-lactoglobulin B and forming new peptides. It is also known that this organism is highly proteolytic (Shihata & Shah, 2000). *B. thermophilum* 20210 also showed a preference for the fraction B of β-lactoglobulin where there was a significant decrease in peak size observed during the first 4 hours of incubation when compared to that of the control milk (Table 6.2). *L. delbrueckii* ssp. *bulgaricus* 2501 was observed to be the most active towards utilising β-lactoglobulin B when compared to the other yoghurt and probiotic bacteria studied. There was an increase in β-lactoglobulin B peak size observed in *L. delbrueckii* ssp. *bulgaricus* 2515 and *L. acidophilus* 2405 cultures at 12 hours which indicates that they are actively breaking down milk proteins available in their medium, breaking down existing bonds within the protein fraction to form new peptide linkages.

When β-lactoglobulin B content was quantified in mixed bacterial combinations of *L. acidophilus* 2415 + *Bifidobacterium thermophilum* 20210 and *S. thermophilus* 2014 + *L. delbrueckii* ssp. *bulgaricus* 2515, an increase in the peak size corresponding to the B fraction of the whey protein was observed during the first 4 hours of incubation when compared to that of the control milk (Figure 6.26). These combinations produced 1.33 and 1.55 mg/mL respectively, as opposed to 0.99 mg/mL in control milk. The peak size corresponding to the β-lactoglobulin B fraction decreased in size at 12 hours in the bacterial combinations of *S. thermophilus* + mix and that of *L. acidophilus* 2415 + *B. thermophilum* 20210, which indicates active utilisation of the whey protein and forming new peptide bonds. The rest of the mixed combinations appeared to be either growing at a slower rate or did not need any nutrients at that stage of their growth, where β-lactoglobulin B peak size remained unchanged.

Various trends can be seen in the amount of β-lactoglobulin B content in the starter culture bacterial strains during the first 4 hours of incubation as observed in Figure 6.27.

There was a significant ($P<0.015$) decrease in β-lactoglobulin B peak size demonstrated by BB-12 and T-1 BB showing active growth occurring and new peptide linkages being formed in the process. A significant increase was also shown by T-1 ST and Y-1 ST bacterial cultures (Figure 6.27). After 6 hours of incubation, the peak size of β-lactoglobulin B increased in the cultures of T-1 ST, T-1 LA, Y-1 ST, Y-2 LB, and T-4 ST. On the other hand, the weakly proteolytic T-1 BB and BB-12 were utilising the available whey protein at 12 hours where they demonstrated no change in the peak size corresponding to the β-lactoglobulin-B fraction.

6.4 Conclusion

In the present study, extensive casein degradation and changes in the electrophoretic pattern of the proteins present in the milk fractions were observed during fermentation of cultured milks at 37°C. In general terms proteolysis was faster in milk samples that were fermented by single and mixed bacterial cultures and slower in milks fermented with yoghurt and probiotic strains isolated from dairy starter culture bacteria. All casein fractions incubated with the intracellular samples were digested at a faster rate than that in cell wall extracts.

Most bacterial strains showed 0% hydrolysis of α-casein, β-casein, and κ-casein at the cell wall level when compared to that at the intracellular level. *S. thermophilus* 2002 degraded α_s-casein and β-casein at the fastest rate when compared to other single bacterial cultures studied. Similar results were observed with *S. thermophilus* 2014 except for its action towards κ-casein showing a preference for hydrolysis at the intracellular level.

Both *L. delbrueckii* ssp. *bulgaricus* 2501 and 2515 readily attacked all casein fractions, particularly at the intracellular level. Most of the hydrolysis occurred after 4 hours of incubation. *L. acidophilus* (strains 2405 and 2415) also attacked the casein fractions at the intracellular level, where 64%, 70%, and 82% hydrolysis of α-, β-, and κ-casein, occurred, respectively.

On the other hand, *B. thermophilum* 20210, and *B. infantis* 1912 did show caseinolytic activity, but slightly a different pattern to that observed with the other bacterial strains tested. *B. infantis* 1912 appeared to show casein hydrolysis at both cell levels; however the rate of hydrolysis was slower than that of the other probiotic strain and yoghurt strains. *B. thermophilum* 20210 grows faster than that of *B. infantis* 1912 where hydrolysis of the casein fractions started as early as after 2 hours of incubation. The bovine casein group (α_s, β, and κ-casein) and the whey proteins (β-lactoglobulin and α-lactalbumin) were separated and quantified at the same time using reverse-phase HPLC. *S. thermophilus* 2014 and *L. acidophilus* 2405 showed a preference for α_s-casein degradation when compared to the other bacterial single cultures studied.

On the other hand, *Bifidobacterium* ssp. (1941 and 20099) appeared to hydrolyse α_s-casein at a slower rate. *B. thermophilum* (20210) appeared to break the α-casein fraction into a smaller peptide, which indicates that it actively degraded the casein fraction at a faster rate than the other *Bifidobacterium* ssp. strains, studied, thus the smaller peak size. It appears that the combination of yoghurt bacteria in a mixed culture did degrade α-casein at a faster rate than when cultured alone as would have been expected, indicating they actively break down existing bonds and form smaller peptide fractions. The other yoghurt bacteria, *L. delbrueckii* ssp. *bulgaricus* 2501 and 2515 did not appear to degrade β-casein at the same rate as that exhibited by *S. thermophilus* strains. *L. delbrueckii* ssp. *bulgaricus* 2501 was able to break down peptide bonds within the κ-casein fraction of milk proteins. It was observed that all mixed bacterial cultures studied degraded κ-casein to some extent, when compared to that of κ-casein in the control milk. There was no particular trend observed for α-lactalbumin utilisation by the mixed bacterial cultures.

Table 6.1 Percentage amounts of α_s-, β-, and κ-casein fractions hydrolysed by yoghurt and probiotic bacteria in RSM at 37°C

Bacterial culture	Casein fraction	Time of incubation (hours)					
		Cell wall			Cell free		
		2	4	6	2	4	6
S. thermophilus 2002	α_s-	0	0	0	25	53	23
	β-	0	0	0	30	66	19
	κ-	100	100	100	100	100	100
S. thermophilus 2014	α_s-	22	21	32	0	0	0
	β-	0	0	0	22	20	29
	κ-	0	0	0	51	56	47
L. delbrueckii ssp. *bulgaricus* 2501	α_s-	0	0	0	0	4	11
	β-	0	0	0	0	2	17
	κ-	0	0	0	0	20	47
L. delbrueckii ssp. *bulgaricus* 2515	α_s-	0	0	3	23	19	23
	β-	0	0	1	0	0	0
	κ-	0	0	0	8	19	33
L. acidophilus 2405	α_s-	0	0	0	28	5	64
	β-	0	0	0	29	35	70
	κ-	0	36	30	58	3	82
L. acidophilus 2415	α_s-	10	0	0	0	0	5
	β-	15	0	0	0	0	3
	κ-	48	0	0	0	0	13
B. infantis 1912	α_s-	0	9	100	0	16	0
	β-	22	41	30	0	3	17
	κ-	0	26	0	0	0	0
B. thermophilum 20210	α_s-	38	57	48	15	30	5
	β-	21	12	24	7	7	19
	κ-	53	69	33	17	34	0

Table 6.2 Amount of casein and whey protein quantitated in dairy farmer milk (control) at 0 hours.

Protein fraction	mg/mL
α-casein	7.71 ± 0.30
β-casein	7.74 ± 0.34
κ-casein	7.18 ± 0.05
α-lactalbumin	0.25 ± 0.01
β-lactoglobulin A	1.09 ± 0.05
β-lactoglobulin B	1.00 ± 0.03

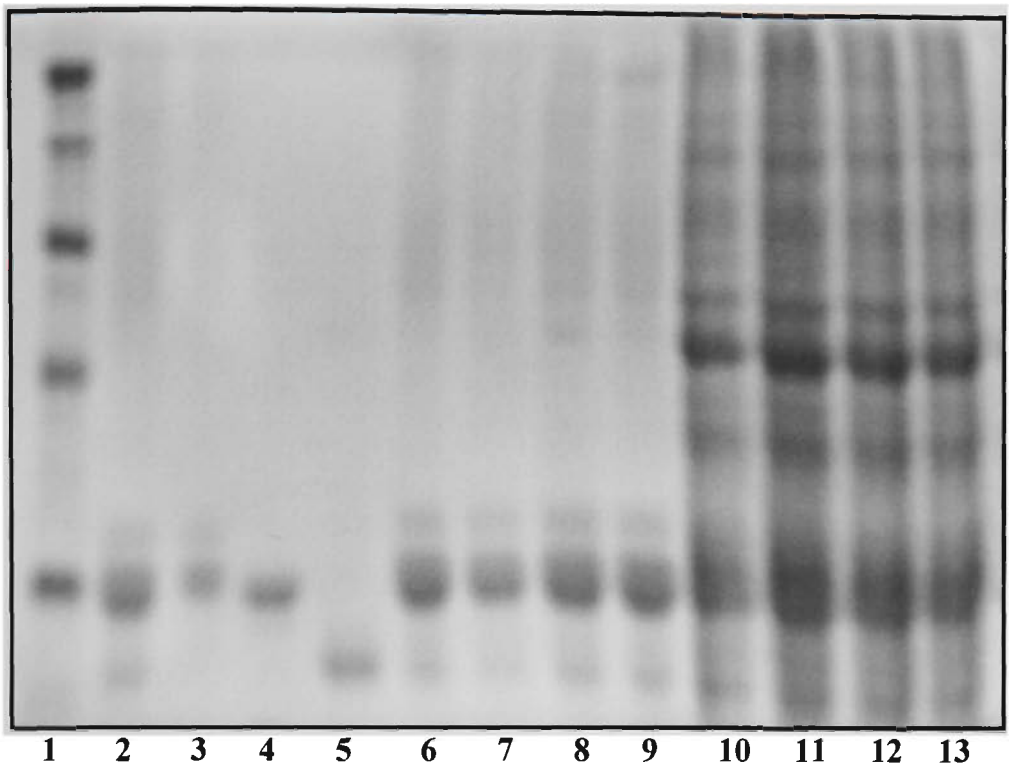

Figure 6.1 Coomassie blue stain of SDS-PAGE of CWE and CFE samples collected from ST 2002. Lane 1=prestained broad range molecular markers; lane 2=casein standard; lane 3= α -casein standard; lane 4= β-casein standard; lane 5=κ-casein standard; lanes 6, 7, 8, and 9=CW extracts of ST 2002 at 0, 2, 4, and 6 hours; and lanes 10, 11, 12, and 13=CF extracts of ST 2002 at 0, 2, 4, and 6 hours.

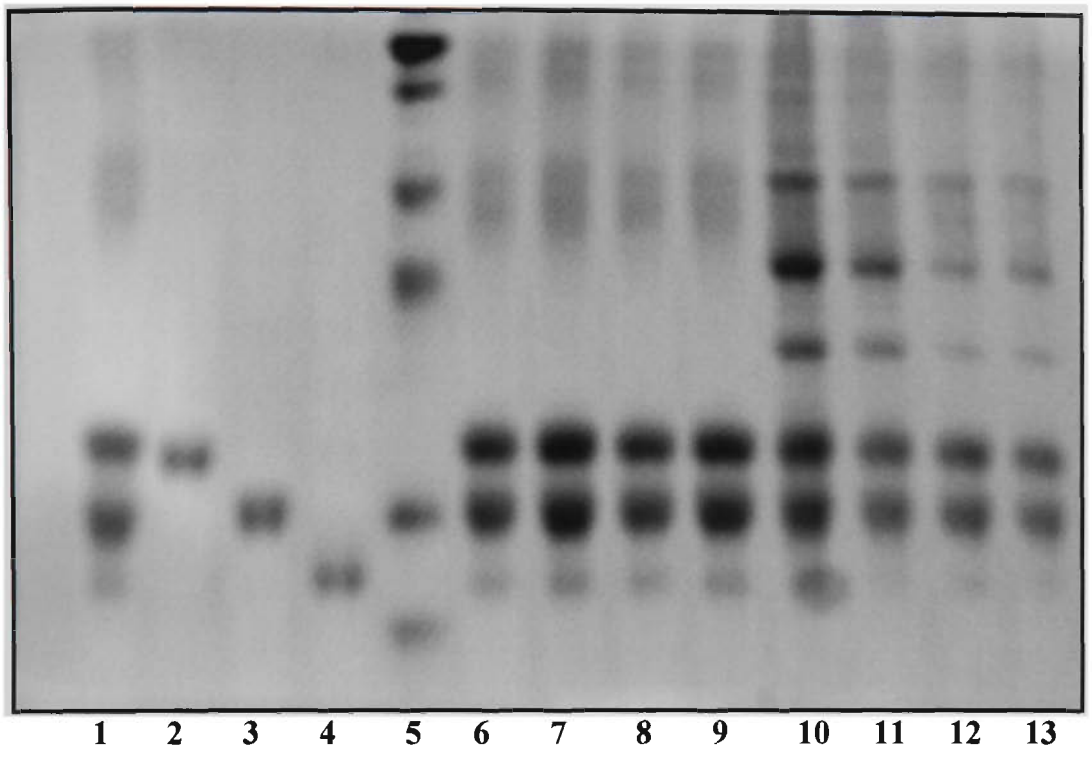

Figure 6.2 Coomassie blue stain of SDS-PAGE of CWE and CFE samples collected from ST 2014. Lane 1=casein standard; lane 2= α -casein standard; lane 3= β-casein standard; lane 4=κ-casein standard; lane 5= prestained broad range molecular markers; lanes 6, 7, 8, and 9=CW extracts of ST 2014 at 0, 2, 4, and 6 hours; and lanes 10, 11, 12, and 13=CF extracts of ST 2014 at 0, 2, 4, and 6 hours.

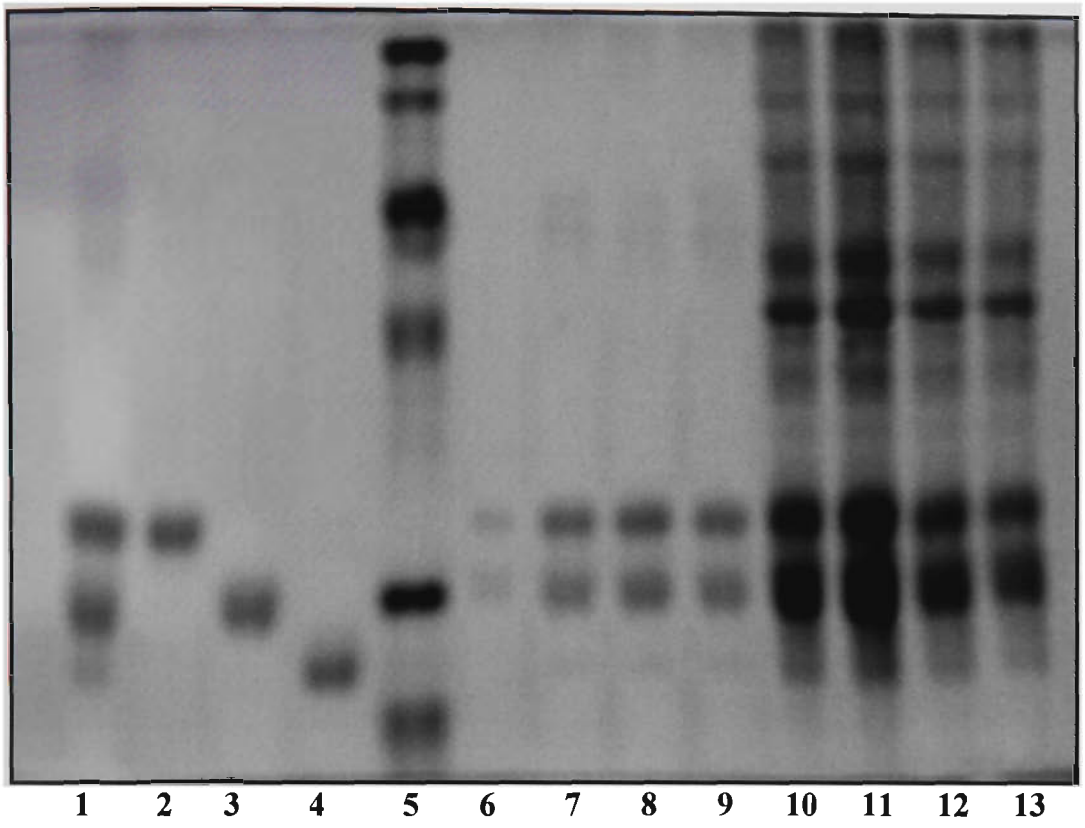

Figure 6.3 Coomassie blue stain of SDS-PAGE of CWE and CFE samples collected from LB 2501. Lane 1=casein standard; lane 2= α -casein standard; lane 3= β-casein standard; lane 4=κ-casein standard; lane 5= prestained broad range molecular markers; lanes 6, 7, 8, and 9=CW extracts of LB 2501 at 0, 2, 4, and 6 hours; and lanes 10, 11, 12, and 13=CF extracts of LB 2501 at 0, 2, 4, and 6 hours.

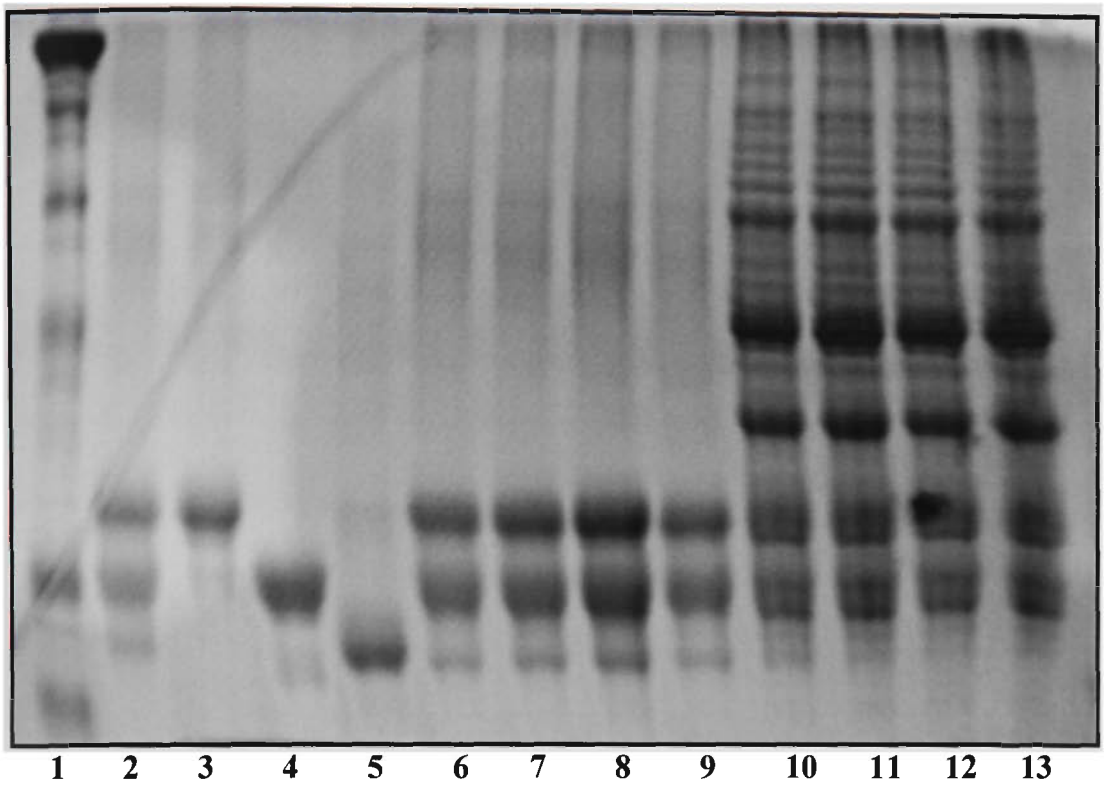

Figure 6.4 Coomassie blue stain of SDS-PAGE of CWE and CFE samples collected from LB 2515. Lane 1=prestained broad range molecular markers; lane 2=casein standard; lane 3= α -casein standard; lane 4= β-casein standard; lane 5=κ-casein standard; lanes 6, 7, 8, and 9=CW extracts of LB 2515 at 0, 2, 4, and 6 hours; and lanes 10, 11, 12, and 13=CF extracts of LB 2515 at 0, 2, 4, and 6 hours.

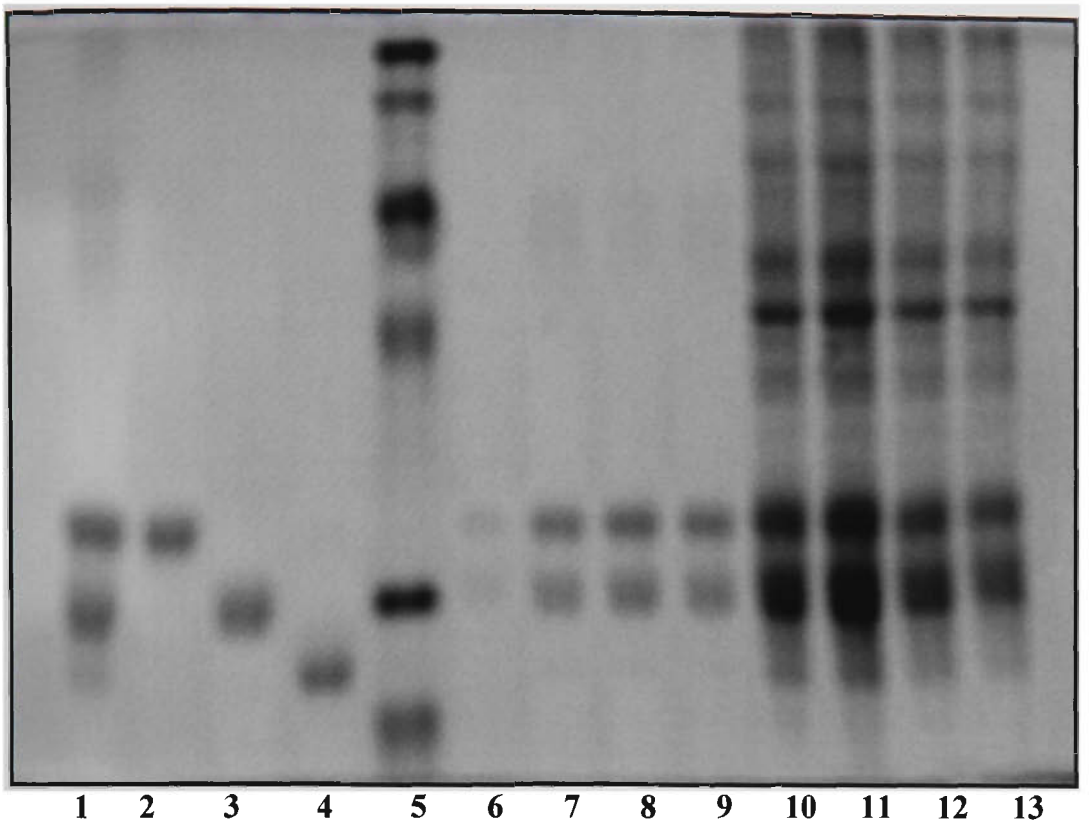

Figure 6.5 Coomassie blue stain of SDS-PAGE of CWE and CFE samples collected from LA 2401. Lane 1=casein standard; lane 2= α -casein standard; lane 3= β-casein standard; lane 4=κ-casein standard; lane 5= prestained broad range molecular markers; lanes 6, 7, 8, and 9=CW extracts of LA 2401 at 0, 2, 4, and 6 hours; and lanes 10, 11, 12, and 13=CF extracts of LA 2401 at 0, 2, 4, and 6 hours.

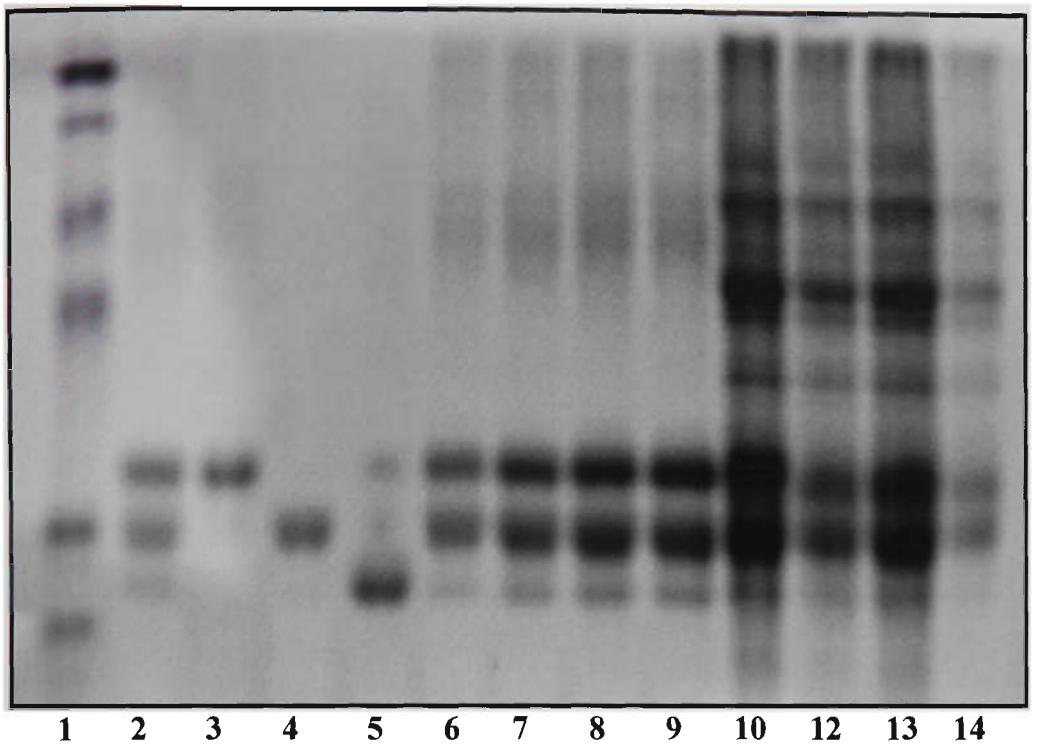

Figure 6.6 Coomassie blue stain of SDS-PAGE of CWE and CFE samples collected from LA 2405. Lane 1=prestained broad range molecular markers; lane 2=casein standard; lane 3= α -casein standard; lane 4= β-casein standard; lane 5=κ-casein standard; lanes 6, 7, 8, and 9=CW extracts of LA 2405 at 0, 2, 4, and 6 hours; and lanes 10, 11, 12, and 13=CF extracts of LA 2405 at 0, 2, 4, and 6 hours.

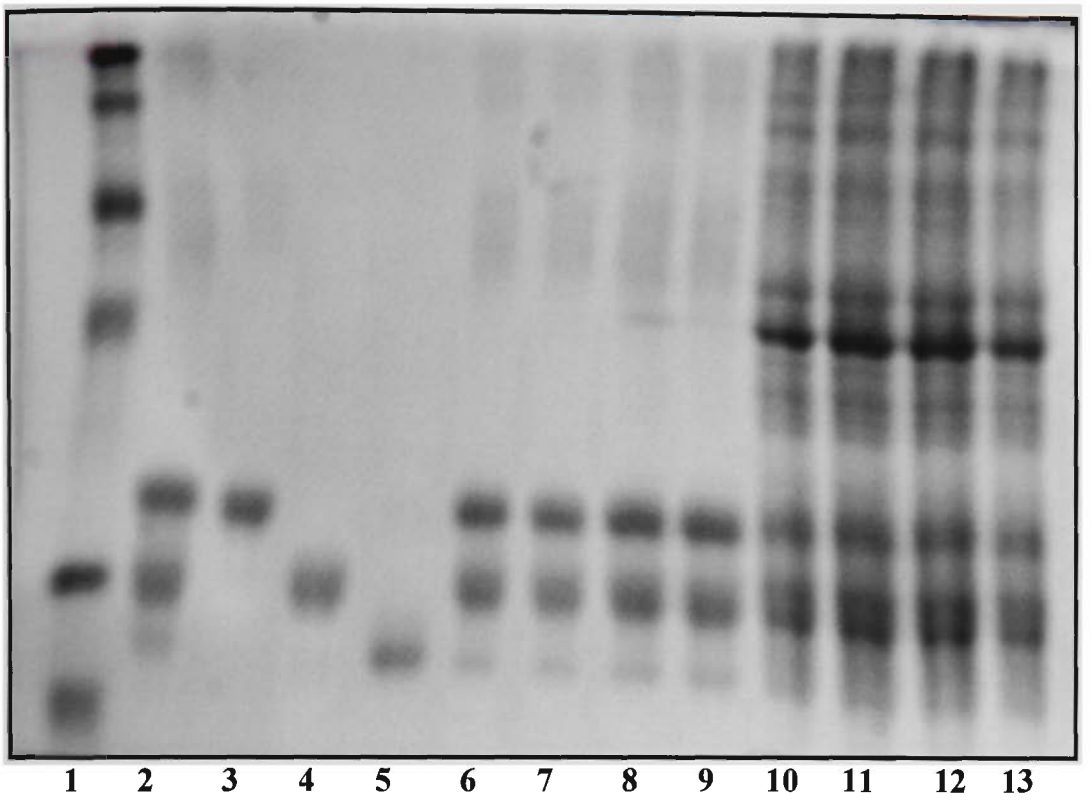

Figure 6.7 Coomassie blue stain of SDS-PAGE of CWE and CFE samples collected from LA 2415. Lane 1=prestained broad range molecular markers; lane 2=casein standard; lane 3= α -casein standard; lane 4= β-casein standard; lane 5=κ-casein standard; lanes 6, 7, 8, and 9=CW extracts of LA 2415 at 0, 2, 4, and 6 hours; and lanes 10, 11, 12, and 13=CF extracts of LA 2415 at 0, 2, 4, and 6 hours.

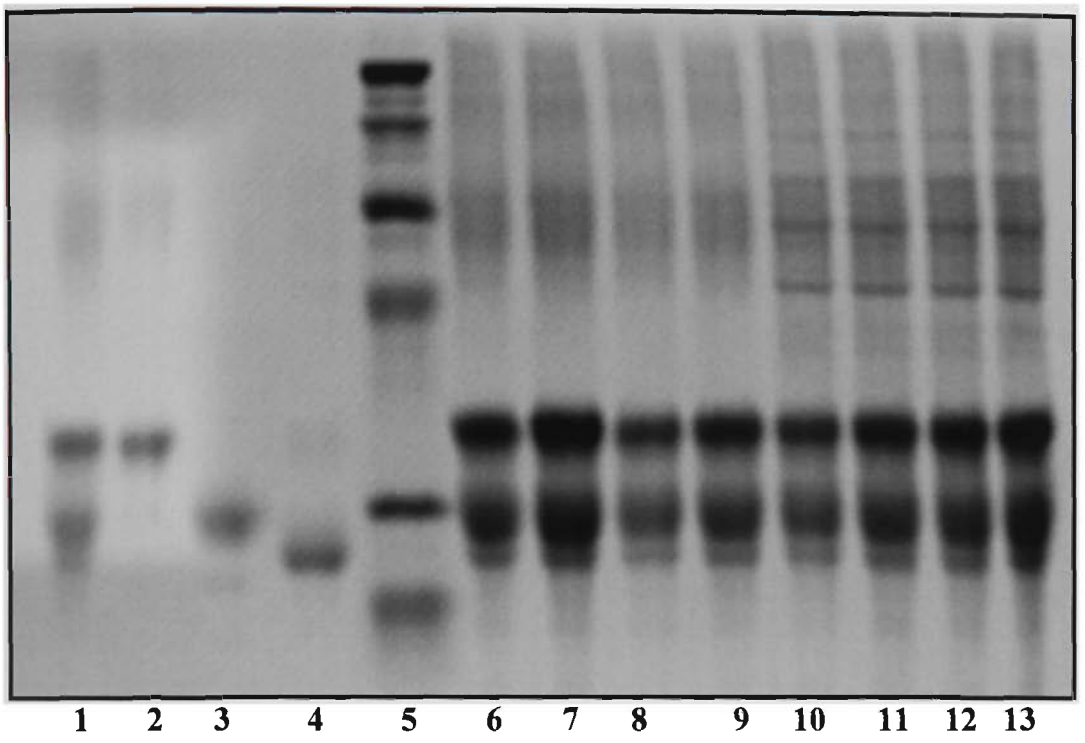

Figure 6.8 Coomassie blue stain of SDS-PAGE of CWE and CFE samples collected from BB 1912. Lane 1=casein standard; lane 2= α -casein standard; lane 3= β-casein standard; lane 4=κ-casein standard; lane 5= prestained broad range molecular markers; lanes 6, 7, 8, and 9=CW extracts of BB 1912 at 0, 2, 4, and 6 hours; and lanes 10, 11, 12, and 13=CF extracts of BB 1912 at 0, 2, 4, and 6 hours.

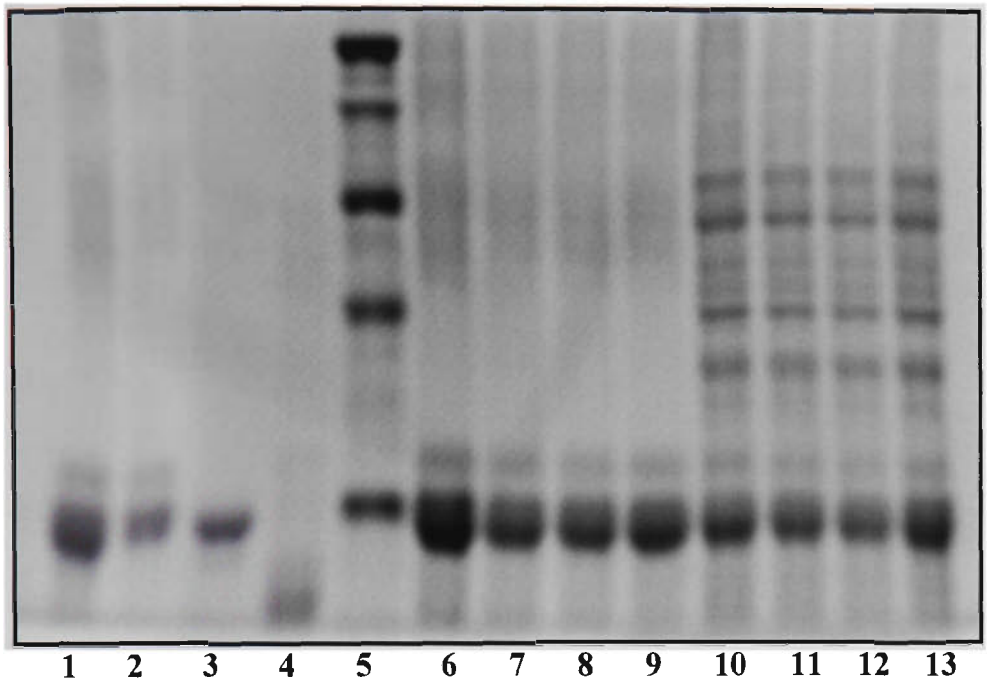

Figure 6.9 Coomassie blue stain of SDS-PAGE of CWE and CFE samples collected from BB 20210. Lane 1=casein standard; lane 2= α -casein standard; lane 3= β-casein standard; lane 4=κ-casein standard; lane 5= prestained broad range molecular markers; lanes 6, 7, 8, and 9=CW extracts of BB 20210 at 0, 2, 4, and 6 hours; and lanes 10, 11, 12, and 13=CF extracts of BB 20210 at 0, 2, 4, and 6 hours.

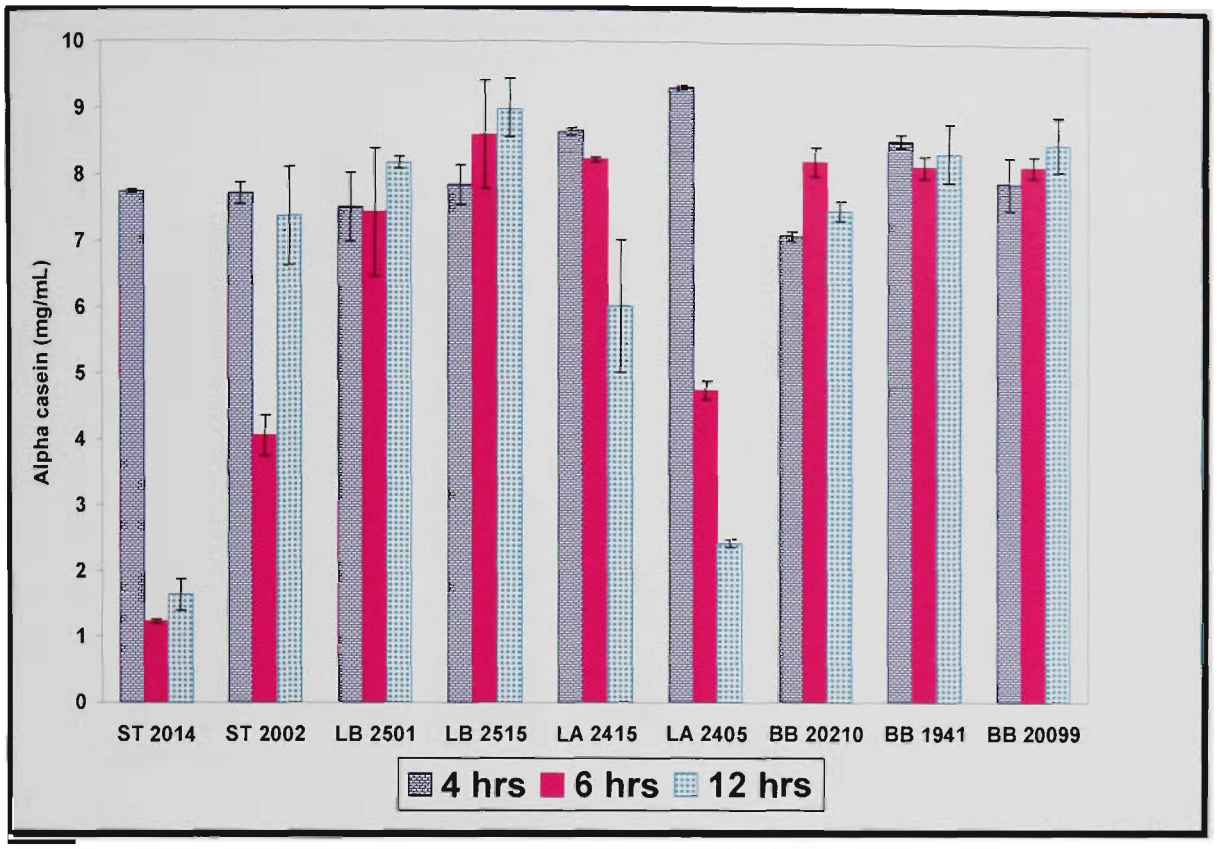

Figure 6.10 Utilization of α_s-casein (mg/mL) by single yoghurt and probiotic bacterial cultures in RSM.

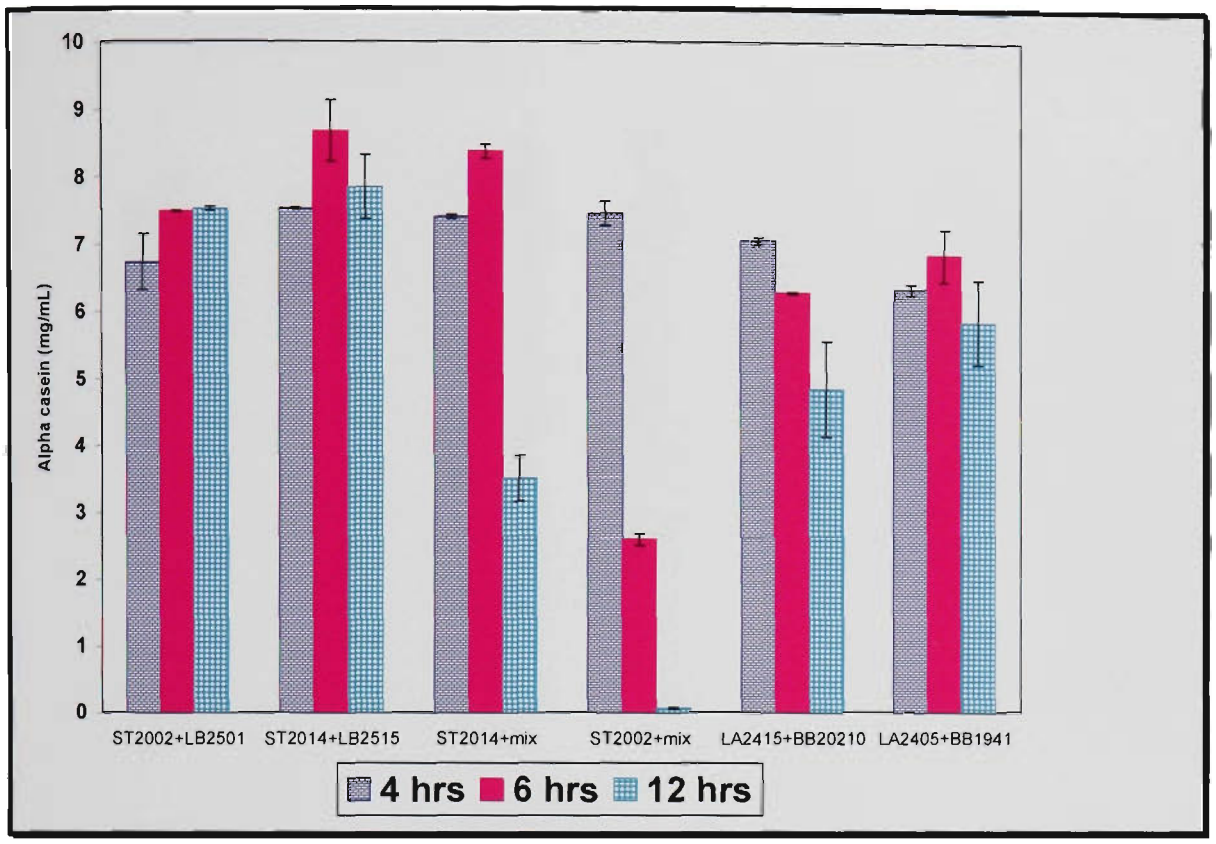

Figure 6.11 Utilization of α_s-casein (mg/mL) by mixed yoghurt and probiotic bacterial cultures in RSM.

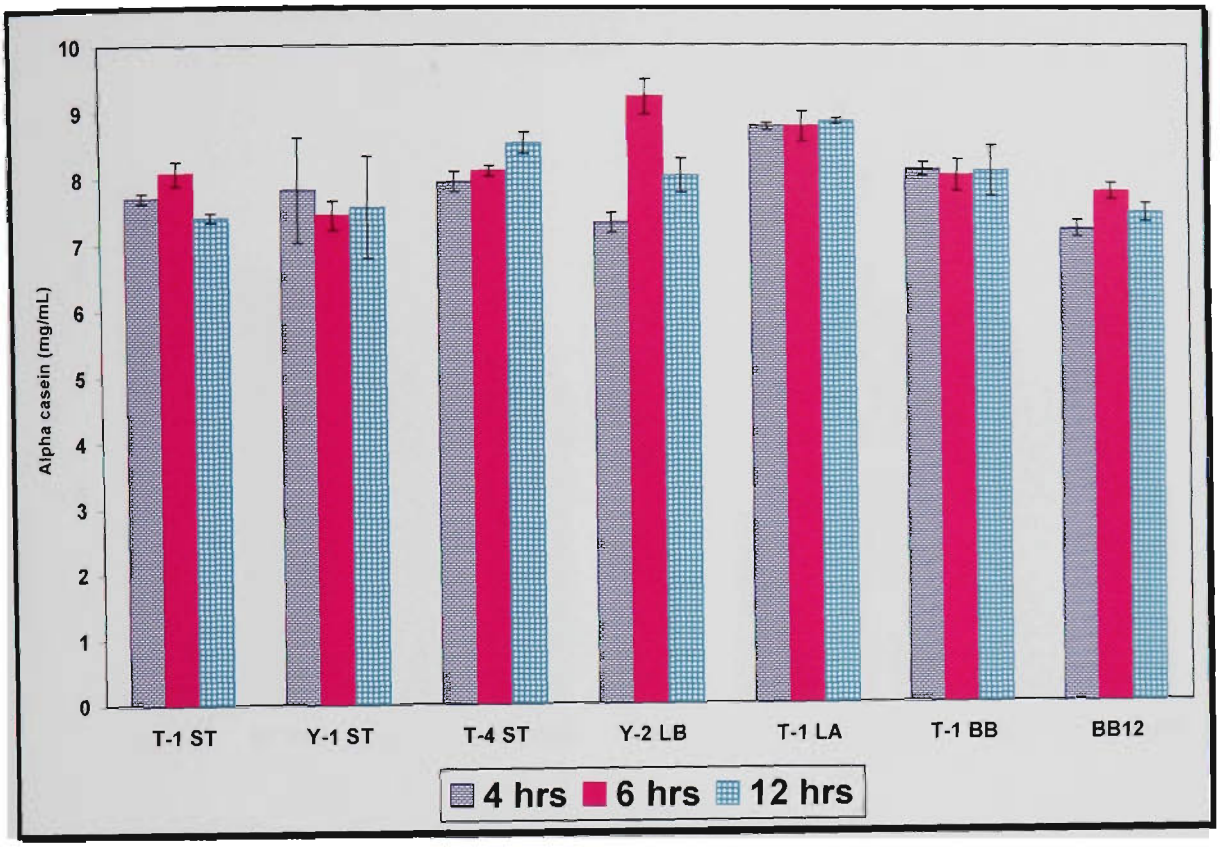

Figure 6.12 Utilization of α_s-casein (mg/mL) by starter culture bacterial strains in RSM.

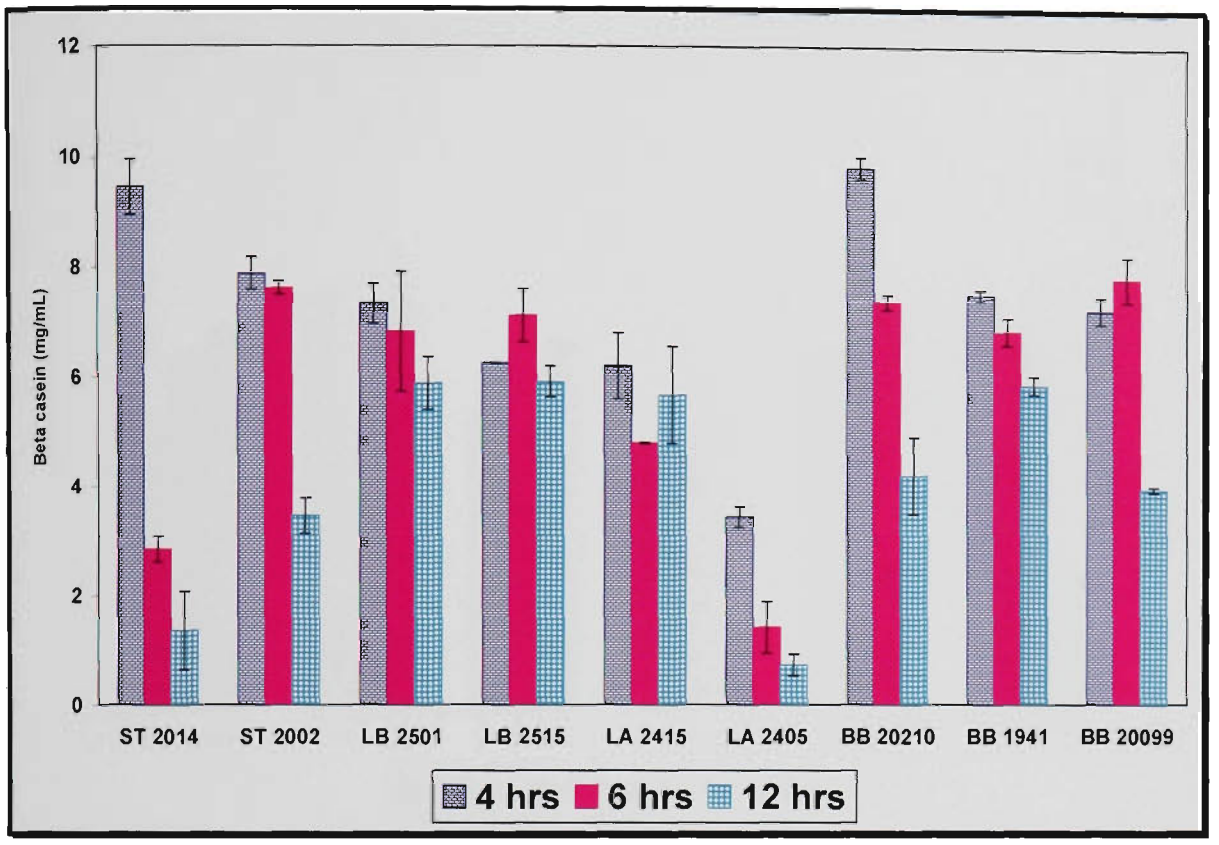

Figure 6.13 Utilization of β-casein (mg/mL) by single yoghurt and probiotic bacterial cultures in RSM.

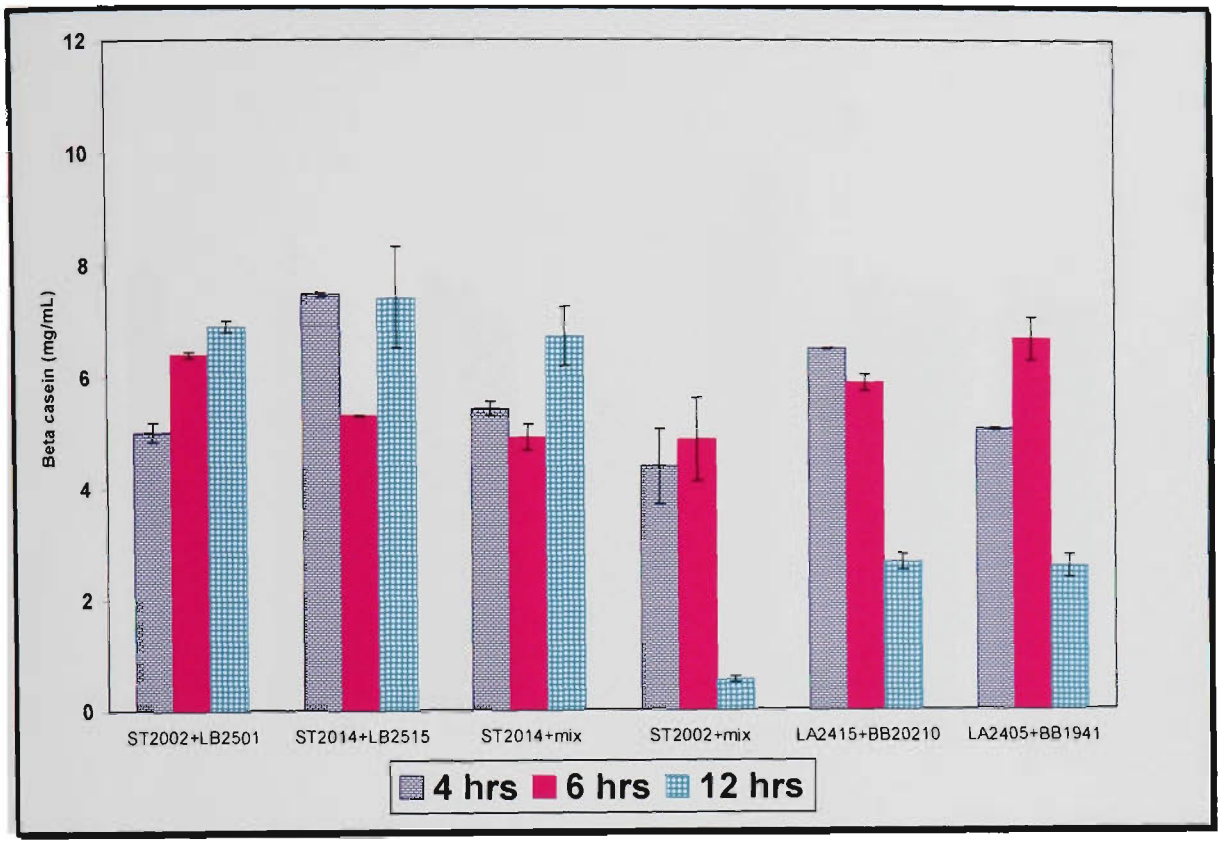

Figure 6.14 Utilization of β-casein (mg/mL) by mixed yoghurt and probiotic bacterial cultures in RSM.

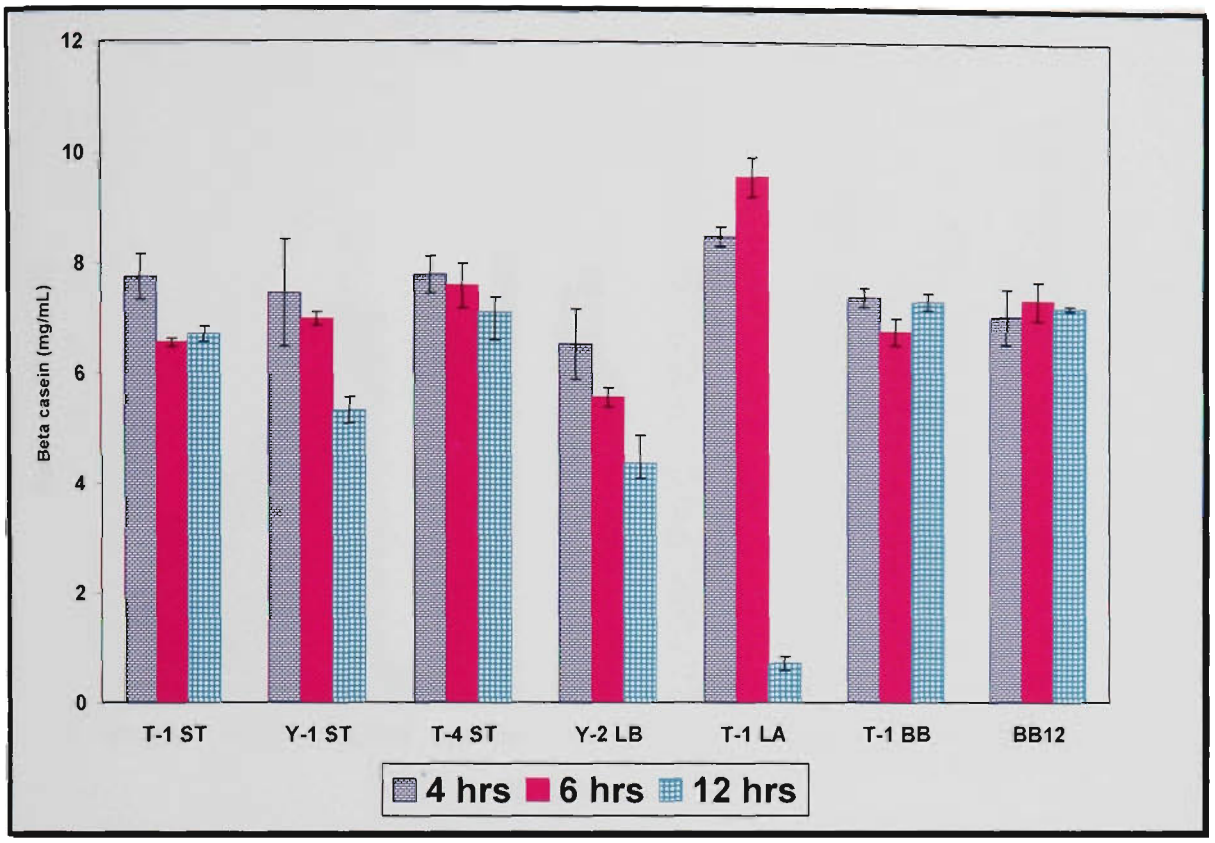

Figure 6.15 Utilization of β-casein (mg/mL) by starter culture bacterial strains in RSM.

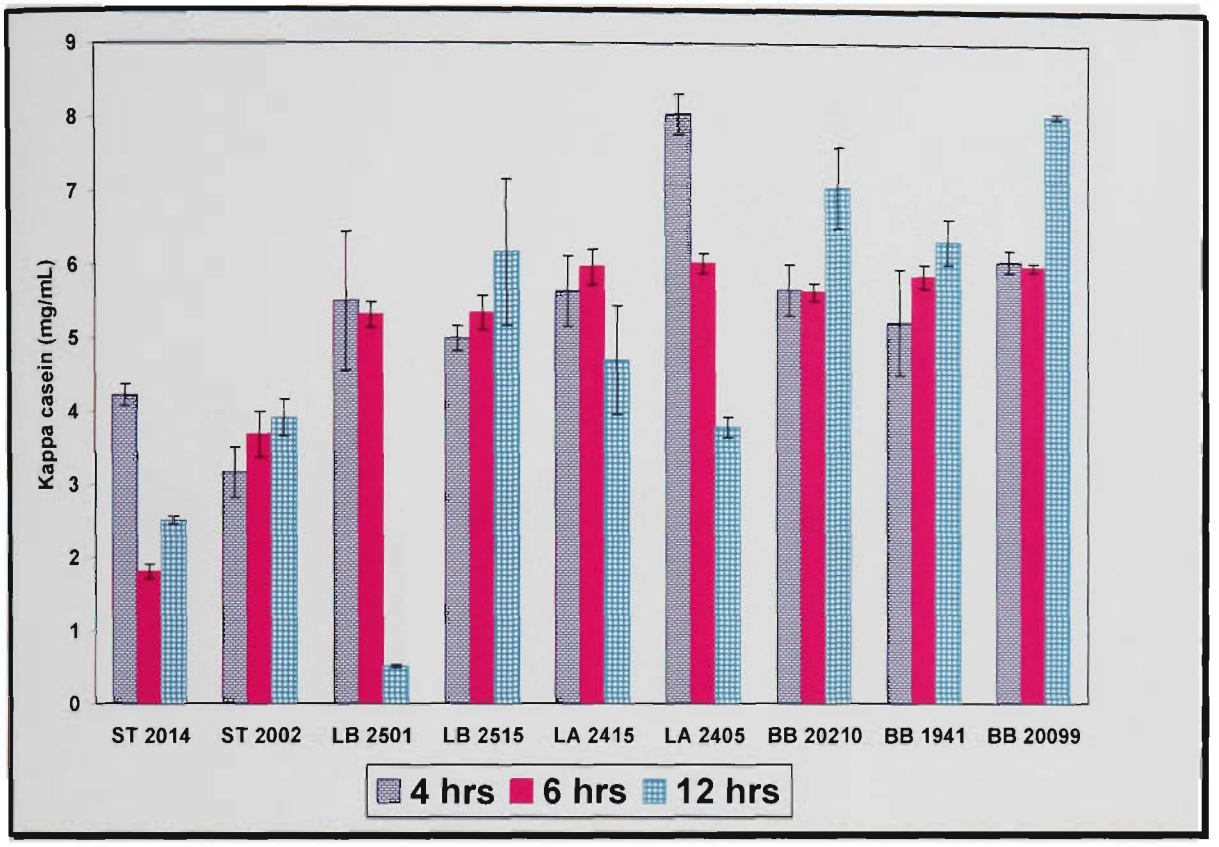

Figure 6.16 Utilization of κ-casein (mg/mL) by single yoghurt and probiotic bacterial cultures in RSM.

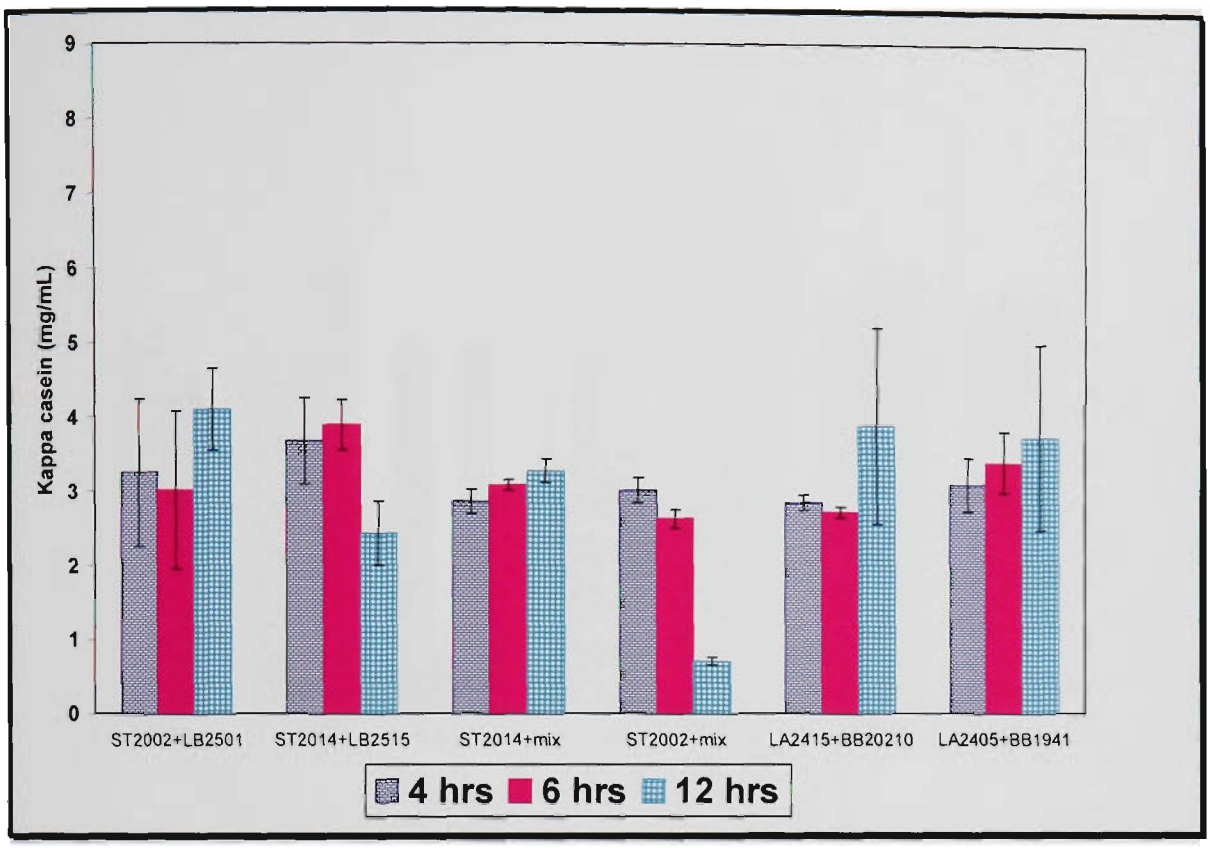

Figure 6.17 Utilization of κ-casein (mg/mL) by mixed yoghurt and probiotic bacterial cultures in RSM.

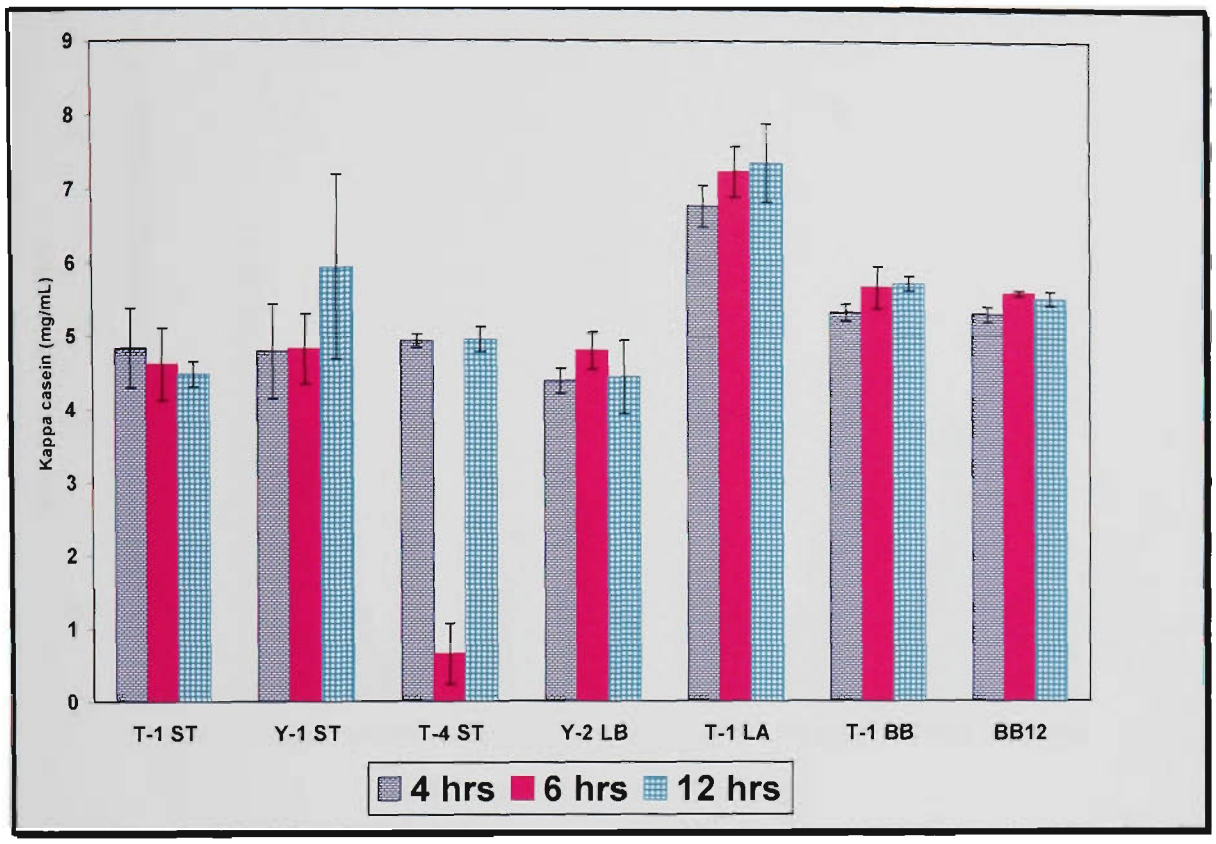

Figure 6.18 Utilization of κ-casein (mg/mL) by starter culture bacterial strains in RSM.

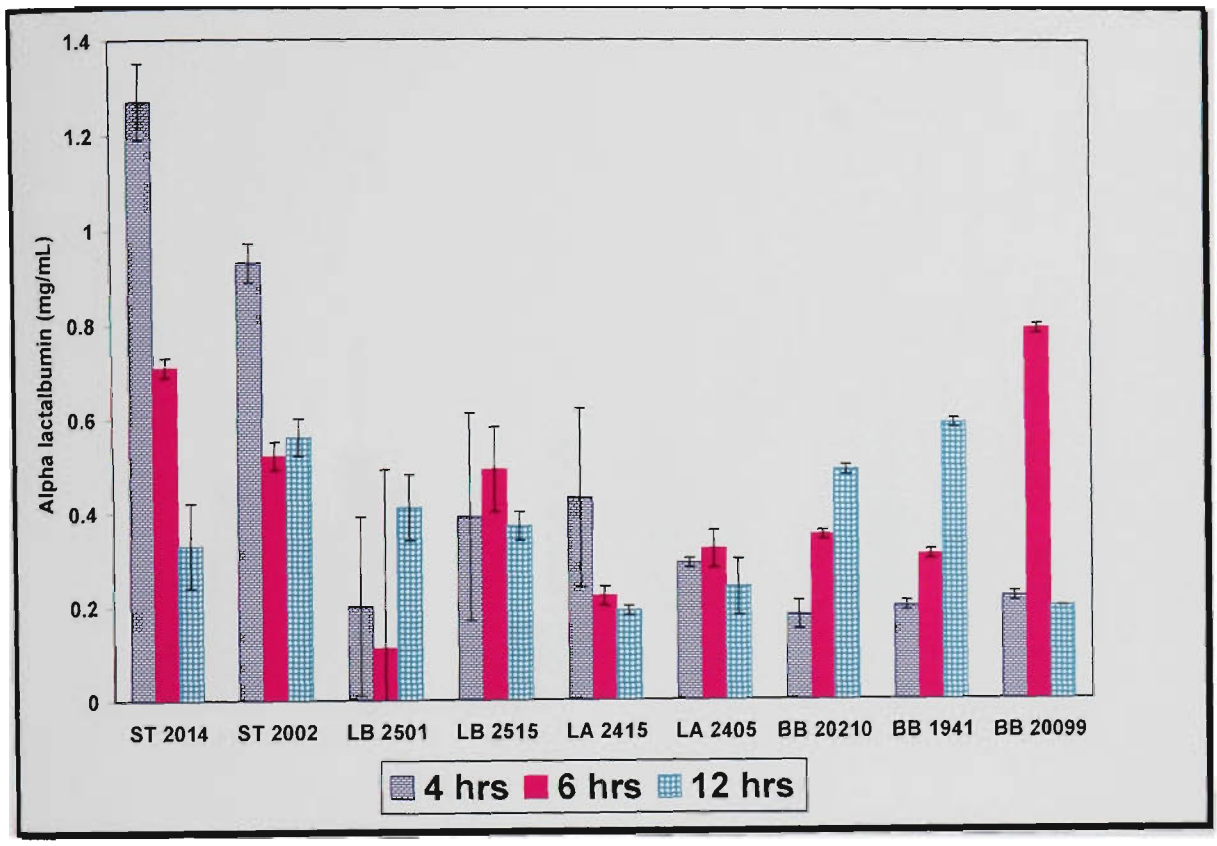

Figure 6.19 Utilization of α-lactalbumin (mg/mL) by single yoghurt and probiotic bacterial cultures in RSM.

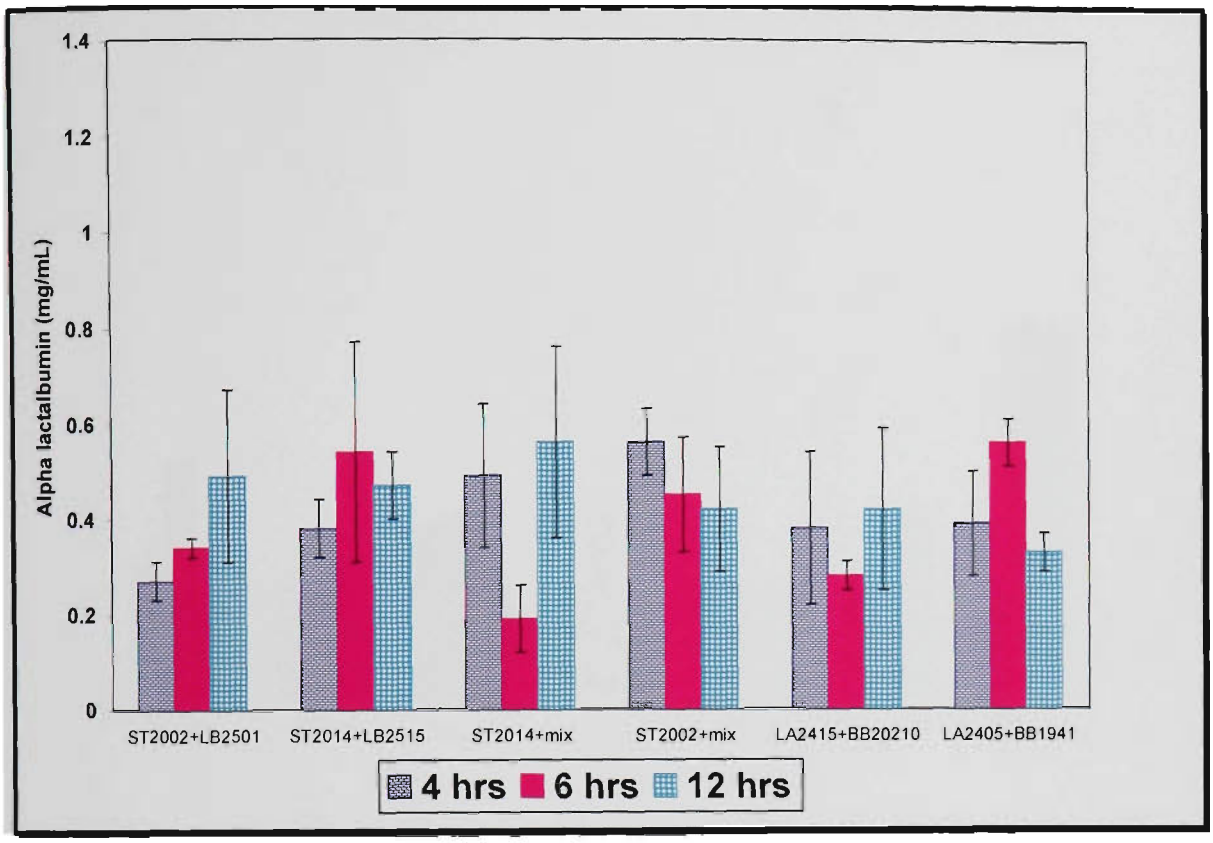

Figure 6.20 Utilization of α-lactalbumin (mg/mL) by mixed yoghurt and probiotic bacterial cultures in RSM.

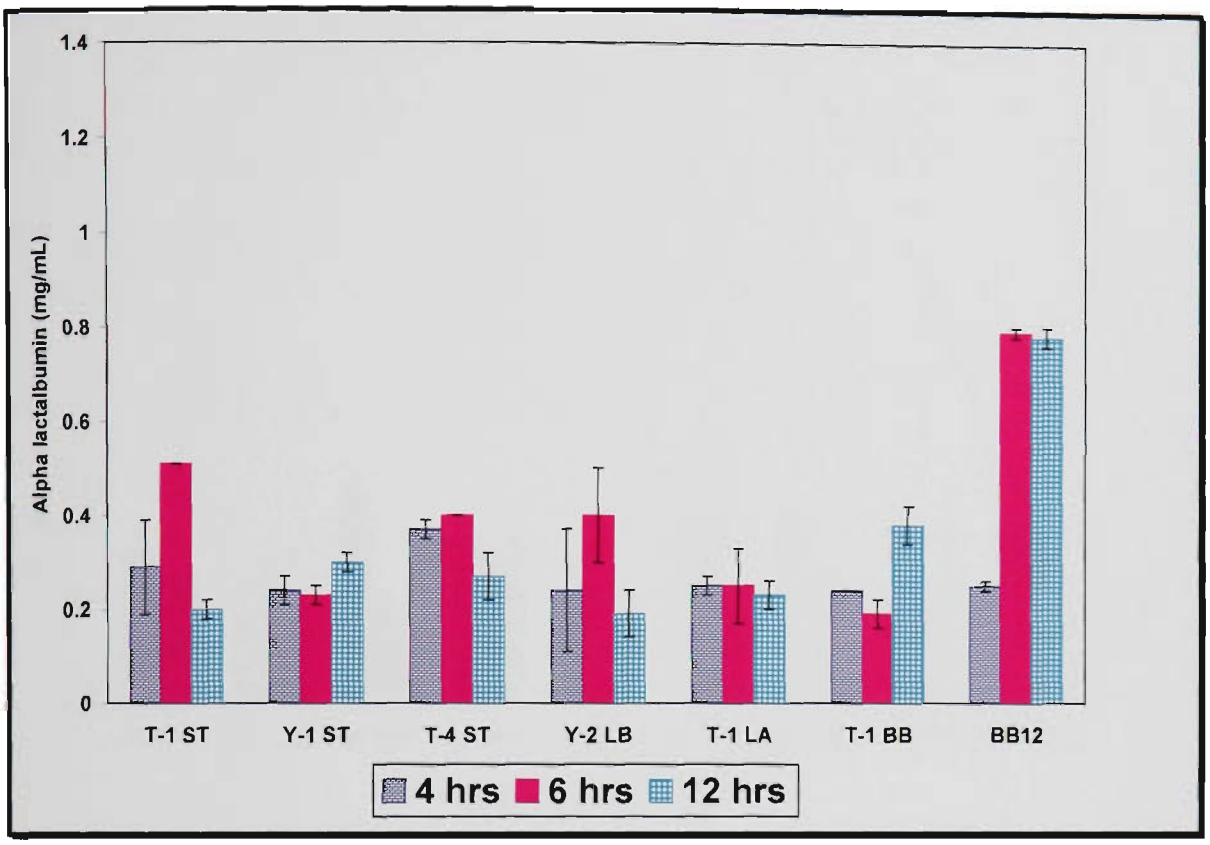

Figure 6.21 Utilization of α-lactalbumin (mg/mL) by starter culture bacterial strains in RSM.

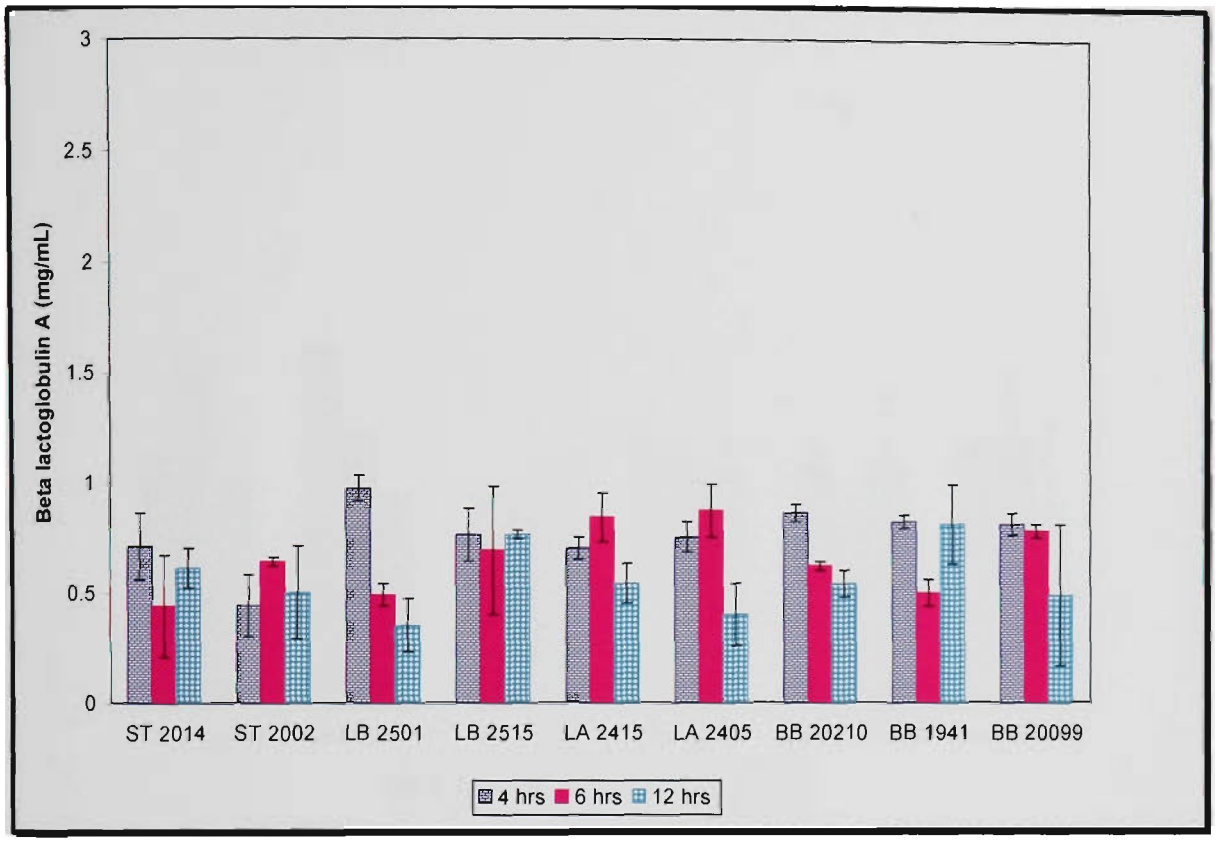

Figure 6.22 Utilization of β-lactoglobulin A (mg/mL) by single yoghurt and probiotic bacterial cultures in RSM.

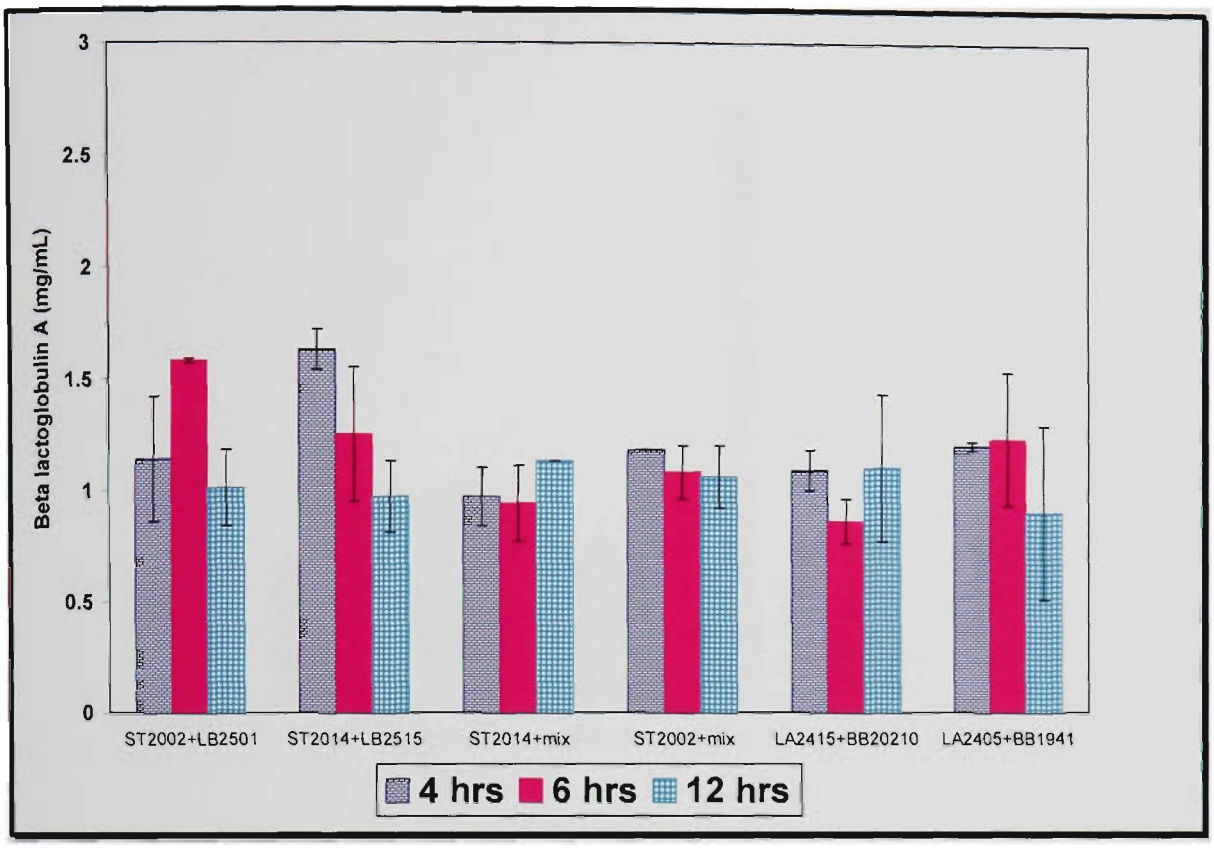

Figure 6.23 Utilization of β-lactoglobulin A (mg/mL) by mixed yoghurt and probiotic bacterial cultures in RSM.

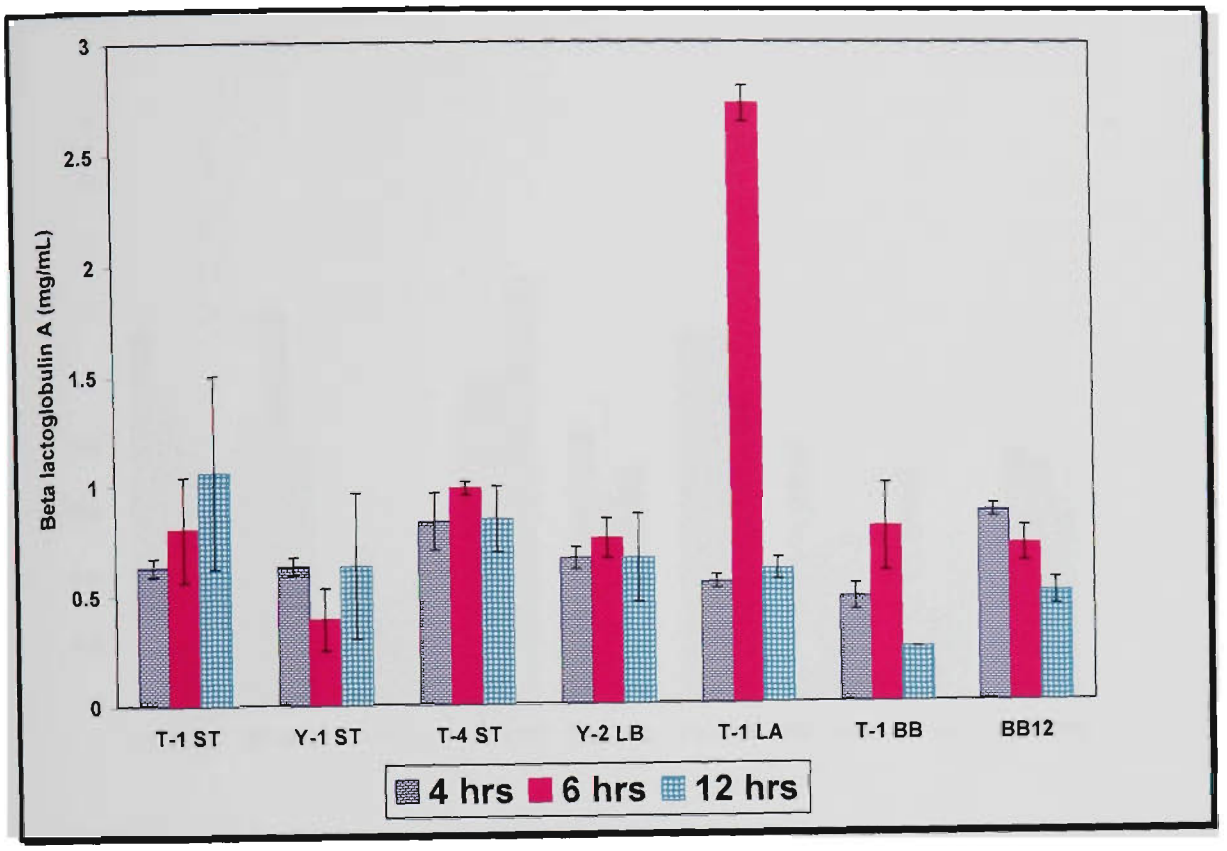

Figure 6.24 Utilization of β-lactoglobulin A (mg/mL) by starter bacterial strains in RSM.

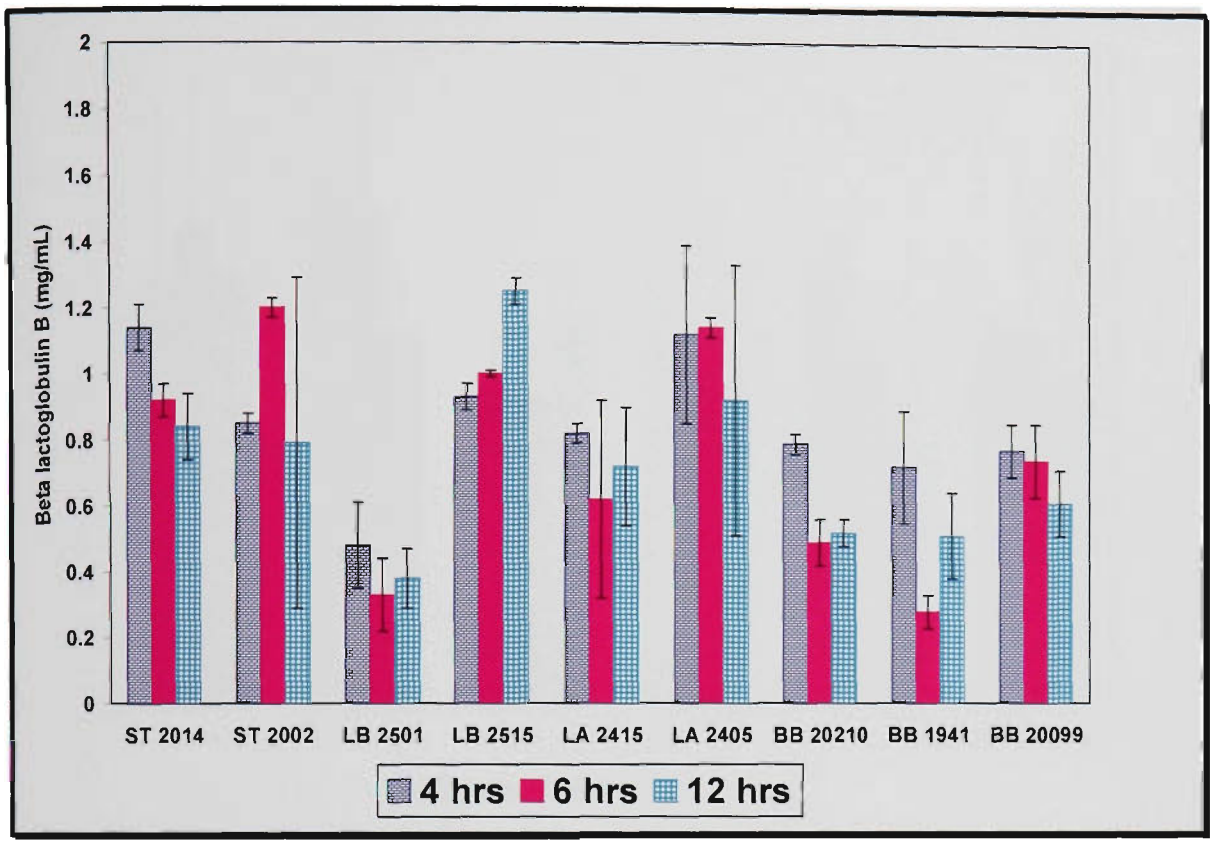

Figure 6.25 Utilization of β-lactoglobulin B (mg/mL) by single yoghurt and probiotic bacterial cultures in RSM.

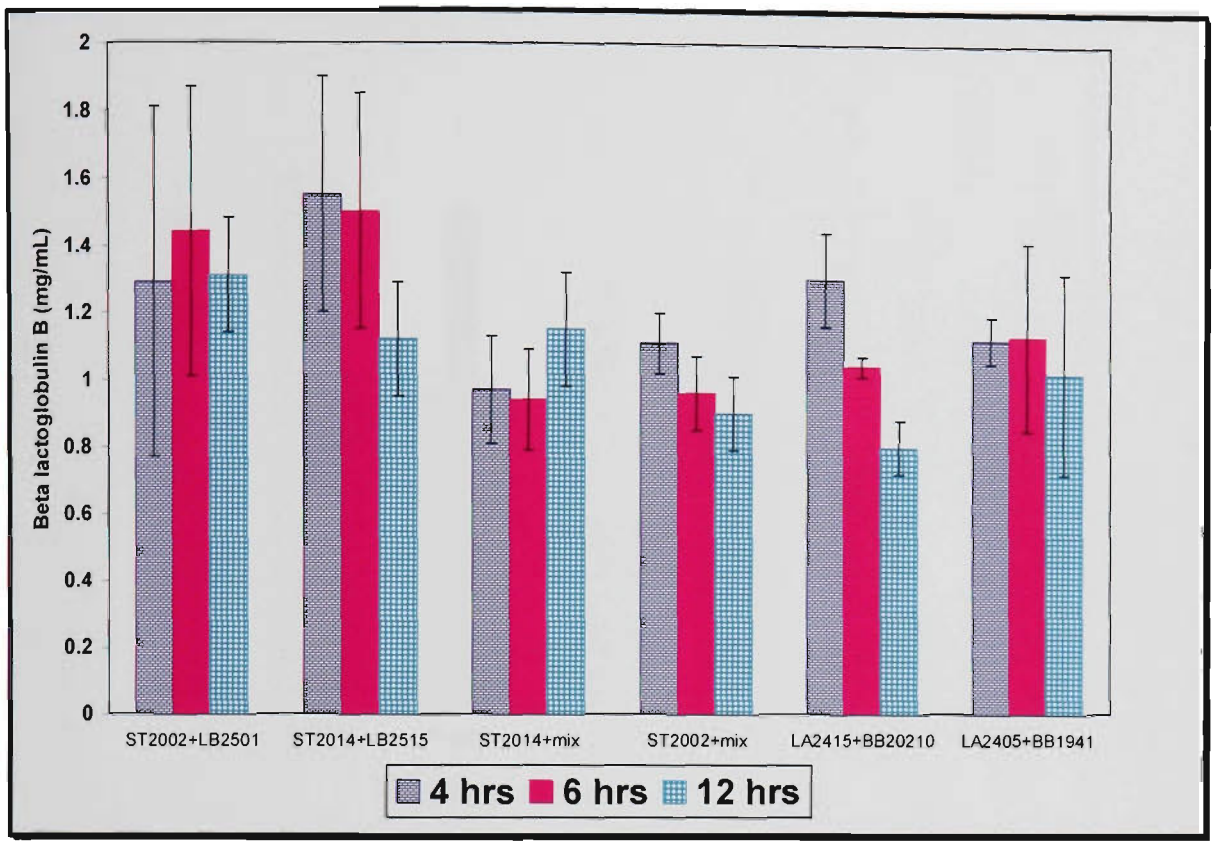

Figure 6.26 Utilization of β-lactoglobulin B (mg/mL) by mixed yoghurt and probiotic bacterial cultures in RSM.

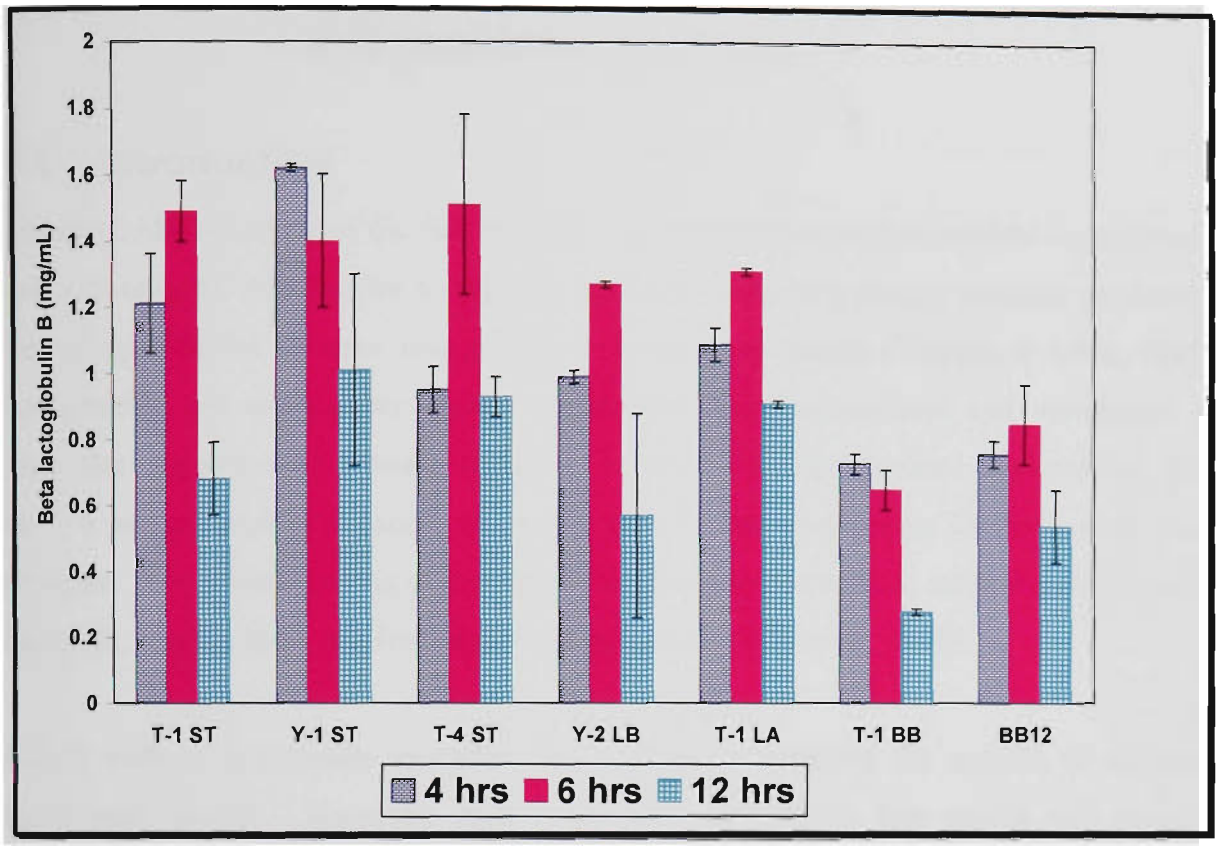

Figure 6.27 Utilization of β-lactoglobulin B (mg/mL) by starter culture strains in RSM.

Chapter 7 Amino acids profile analysis of yoghurt and probiotic bacteria

7.1 Introduction

Among LAB, members of the *Lactobacillus* genus present complex nutrient requirements that can only be satisfied by a culturing medium containing energy sources, precursors for cell-growth and division, and growth stimulatory substances (Thomas & Mills, 1981). Lactobacilli are extensively used in industrial food production and nowadays as functional ingredients for health benefits. Isolated from an intestinal environment, they have a widespread application as, e.g. probiotic microorganisms in fermented dairy products. The understanding of their physiological requirements in natural environments, like milk, enables their use for industrial applications (Elli *et al.*, 1999).

Cow's milk is a naturally complex medium which supports the growth of different lactobacilli species. However, milk is generally low in the free amino acid content. Bacterial growth can be improved in these cases by the addition of substances of undefined composition to the growth medium, such as yeast extract or peptones of various origins. The off-flavours associated with these components, their cost and variability in stimulating bacterial growth render these substances less suitable for, e.g. an industrial production of commercial dairy products. The presence of free amino acids in cow's milk can therefore affect the ability of lactic acid bacteria to develop in this environment (Elli *et al.*, 1999).

The spectrum of free amino acids in milk and yoghurt is dependent on several variables. Milks from different species (cow, sheep and goat) have different contents of amino acids, i.e. $\leq$ 10, 3.78 and 20.6mg/100ml respectively, and in addition, goats' milk has, relative to the others, much higher levels of alanine, glycine, glutamic acid, serine, and threonine. Slightly higher levels of amino acids are obtained when the fermentation is carried out at 42°C for 2-3 hours, rather that at 42°C for 1 hour followed by 5-6 hours at

30-32°C; the total amino acid contents of such yoghurts were 23.6 and 19.4mg/ml (Tamime & Robinson, 1980).

Due to the fact that *L. delbrueckii* ssp. *bulgaricus* is more proteolytic than *S. thermophilus*, the higher the ratio of rods to cocci in the starter culture, the higher the amino acid content is likely to be in the corresponding yoghurt. The temperature of storage of yoghurt can affect the level of free amino acids in the product, i.e. the higher the storage temperature, the greater the increase in free amino acids. Ottogali *et al.* (1974) stored full and low (1%) fat natural yoghurts at 4°C and 20°C for a duration of 60 days, and the increases in the level of amino acids in these yoghurts were 2.36 and 1.00 mg/100mL at 4°C and 7.57 and 14.65mg/100ml respectively at 20°C. The amino acid content of yoghurt is also dependent on the titratable acidity of the product (Tamime & Robinson, 1980).

Yoghurts which contained 1.9 and 1.72-1.73% lactic acid had total amino acid contents of 70 and 41-50mg/100ml respectively. Incidentally, the figure of 70mg/100g of yoghurt is the highest level reported in the literature, and it could be argued that such acidic yoghurt could be the result of prolonged incubation, and hence the amino acid content reflects directly the extent of the metabolic activity of the starter culture (Tamime & Robinson, 1980).

The final amino acid content of yoghurt made from cow's milk may range from 18.7-33mg/100ml, and it is probable that the acidities of these yoghurts were 1.0-1.4% lactic acid. It is important, of course, that the total acid content of yoghurt reflects a balance between proteolysis and assimilation by the bacteria. Some amino acids, such as glutamic acid, proline and, to a lesser degree, alanine and serine, are presumably not required by the yoghurt organisms, and thus accumulate in larger quantities in the product than the remaining amino acids which are utilised by *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* during growth and/or fermentation (Tamime & Deeth, 1980).

The change in pattern of proteolytic activity during lactic acid fermentation is of basic importance for the degree of symbiotic interaction between *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus*. Some LAB do not synthesize essential amino acids. They require an exogenous nitrogen source and utilize peptides and proteins from the growth medium by means of more or less complete enzyme systems (Zourari *et al.*, 1992).

Of the essential amino acids required by most lactic streptococci, only methionine appears to be absent in free form from normal milk. However, methionine is present in low molecular weight peptides contained in the non-protein nitrogen (NPN) fraction. Comparison of the amino acid requirements for bacterial protein synthesis with the levels of free amino acids in milk indicates that most free amino acid levels are well below the minimum requirement for growth to high cell densities (Thomas & Mills, 1981).

Previous studies of Dave and Shah (1998) have found that the addition of whey protein concentrate and acid casein hydrolysate improved the viability of bifidobacteria to variable extents. SDS-PAGE and amino acid analyses suggested that a nitrogen source in the form of peptides and amino acids improved the viability of bifidobacteria. Media used for enumeration of bifidobacteria often incorporate L-cysteine (0.5 to 0.1%) to improve recovery of bifidobacteria (Laroia & Martin, 1991; Teraguchi *et al.*, 1978). Dave and Shah (1997) also studied the effect of L-cysteine on the growth and viability of yoghurt and probiotic bacteria in yoghurt made from four different commercial starter cultures. Viability of bifidobacteria in one of the starter cultures studied was improved when cysteine was incorporated into the yoghurt mix. Cysteine, a sulfur-containing amino acid could improve amino nitrogen as a growth factor while reducing the redox potential, both of which might favour the growth of anaerobic bifidobacteria species. Collins and Hall (1984) also reported improved viability of some bifidobacterial species in RSM containing 0.05% cysteine.

Therefore this study was undertaken to assess the influence of fortifying milk with some amino acids on growth and acid production by *S. thermophilus* 2002, *L. delbrueckii* ssp.

bulgaricus 2501, *L. acidophilus* 2405, and *Bifidobacterium* ssp. 20210 when cultured alone and in co-culture.

7.2 Materials and Methods

7.2.1 Quantification of total amino acids

Single cultures of *S. thermophilus* (2002 and 2014), *L. delbrueckii* ssp. *bulgaricus* (2501 and 2515), *L. acidophilus* (2405 and 2415), *Bifidobacterium* ssp. (20210, and BB 1941), and mixed cultures of *S. thermophilus* 2002 + *L. delbrueckii* ssp. *bulgaricus* 2501, *S. thermophilus* 2014 + *L. delbrueckii* ssp. *bulgaricus* 2515, *L acidophilus* 2415 + *Bifidobacterium* ssp. 20210, *L acidophilus* 2405 + *Bifidobacterium* ssp. 1941, *S. thermophilus* 2002 + *L. delbrueckii* ssp. *bulgaricus* 2501 + *L acidophilus* 2415 + *Bifidobacterium* ssp. 20210, and *S. thermophilus* 2014 + *L. delbrueckii* ssp. *bulgaricus* 2515 + *L acidophilus* 2405 + *Bifidobacterium* ssp. 1941 were inoculated (1%) into 12 % RSM and incubated overnight (18 hours) at 37°C. An uninoculated RSM was used as control.

These bacterial cultures were then prepared for HPLC analysis as per method described in section 3.2.9.1 in Chapter three. Bacterial samples were then analysed for total amino acids using the AccQ-Tag method (SCL Method 22233). These analyses were conducted by State Chemistry Laboratories of Melbourne, Australia.

7.2.2 Amino acid supplementation studies

This experiment was designed to assess the growth and acid development in RSM supplemented with some amino acids. *S. thermophilus* 2002, *L. delbrueckii* ssp. *bulgaricus* 2501, *L. acidophilus* 2415 and *B. thermophilum* 20210 were obtained from the Victoria University Culture Collection as described in section 3.1.5 in Chapter three. These bacterial strains were cultivated and prepared for the amino acid supplementation studies according to usual practice described in section 3.2.1 in Chapter three. Amino acid supplementation studies are described in detail in section 3.2.9.2 in Chapter three.

7.3 Results and Discussion

7.3.1 Total amino acids quantified in fermented yoghurt and probiotic cultures

Total amino acids released by single bacterial strains and in co-culture in RSM were quantified using the AccQ-Tag HPLC method. Tables 7.1, 7.2, and 7.3 represent the quantification of total amino acids released by single bacterial strains of yoghurt bacteria and probiotic bacteria and in co-culture with each other. The amount of amino acids released is expressed in µg/ml. Results obtained with single and mixed bacterial cultures are compared to that of a control RSM (free of bacteria).

The data presented in Table 7.1 shows the quantity of amino acids released by yoghurt and probiotic bacterial cultures when cultured alone in RSM at 37°C. Amino acids were also quantified in a control RSM (uninoculated) so that comparisons can be made between before and after inoculation with the yoghurt and probiotic bacterial cultures. Total amino acids in the uninoculated RSM were 166.9 µg/ml (Table 7.1). Total amino acids quantified in the bacterial cultures were less than that of the control RSM, where the lowest was observed for *L. delbrueckii* ssp. *bulgaricus* 2501 and *L. acidophilus* 2405, 39.8 and 43.2 µg/ml respectively (Table 7.1).

This indicates that the amino acids present in the RSM prior to inoculation were used up by the bacterial cultures during the fermentation process. The amino acid tyrosine is often used as an indication of proteolysis. Therefore, the quantity of tyrosine produced can be related directly to the degree of proteolysis of RSM by the bacterial cultures. *S. thermophilus* 2002 and *B. thermophilum* 20210 released the highest amounts of tyrosine which agrees with the earlier findings of Shihata & Shah (2000). It was also observed that both *S. thermophilus* strains (2002 and 2014) had utilized the amino acids aspartic acid, threonine, serine, glutamic acid, alanine, valine, leucine, and lysine. The rest of the amino acids tested were not utilized to a great extent. Traces of tryptophan were found in the cultural filtrates of *S. thermophilus* strains (Table 7.1). These observations differ from earlier findings of many researchers. According to some authors, *S. thermophilus*

requires glutamic acid, histidine, methionine, cysteine, valine, leucine and tyrosine (Beshkova *et al.*, 1998). On the other hand, Thomas and Mills (1981) reported that methionine is not required by *S. thermophilus* strains while cysteine and tryptophan are usually essential. The differing findings could be due to the different media and strains used in each study. Variability within strains has been observed by many researchers.

L. delbrueckii ssp. *bulgaricus* strains appeared to be very different in their amino acid utilization pattern where *L. delbrueckii* ssp. *bulgaricus* 2501 appeared to utilize amino acids to a greater extent than that of *L. delbrueckii* ssp. *bulgaricus* 2515. Total amino acids quantified in *L. delbrueckii* ssp. *bulgaricus* 2515 filtrates (96.4 μg/ml) was much greater than that of *L. delbrueckii* ssp. *bulgaricus* 2501 (39.8 μg/ml) (Table 7.1). This indicates that *L. delbrueckii* ssp. *bulgaricus* 2515 is more proteolytic than *L. delbrueckii* ssp. *bulgaricus* 2501 because it is forming amino acids rather than utilizing them which mean they are degrading the available nutrients. This agrees with earlier findings of Shihata & Shah (2000) where *L. delbrueckii* ssp. *bulgaricus* 2515 was more proteolytic than *L. delbrueckii* ssp. *bulgaricus* 2501.

There was a greater variability observed between *L. acidophilus* strains where *L. acidophilus* 2405 utilized a greater quantity of amino acids than *L. acidophilus* 2415, 43.2 μg/ml and 137.9 μg/ml, respectively. *L. acidophilus* 2405 had a preference for the utilization for most of the amino acids except for tyrosine and cystine and cysteine. These amino acids were produced by *L. acidophilus* 2405 (Table 7.1). On the other hand, *L. acidophilus* 2415 did not appear to utilize the amino acids to a great extent as observed for *L. acidophilus* 2405, and this could be because *L. acidophilus* 2415 grows at a slower rate and is less proteolytic (Shihata & Shah, 2000). Previous studies with *L. acidophilus* R-26 (now known as *L. johnsonii* NCC 533) has shown that the key amino acids required for its growth included cysteine, alanine, serine, and isoleucine. Although the strain *L. johnsonii* NCC 533 could grow in the absence of alanine, serine or isoleucine, these amino acids strongly stimulated its growth when exogenously supplied (Elli *et al.*, 1999).

The same observations can be made for the *B. thermophilum* 20210 and *B. longum* 1941, where *B. thermophilum* 20210 seemed to utilize amino acids faster than that of *B. longum* 1941 (Table 7.1). When compared to control RSM, *B. thermophilum* 20210 released about 69.9 µg/ml of the total amino acids available compared to 114.6 µg/ml for *B. longum* 1941. This could be due to the fact that *B. thermophilum* 20210 is highly proteolytic in nature and grows faster than *B. longum* 1941 (Shihata & Shah, 2000), so it is utilizing the available amino acids at a faster pace than *B. longum* 1941.

Data in Table 7.2 represents total amino acid content quantified in mixed cultures of yoghurt bacteria and also that of probiotic bacteria. There was not much difference in amino acid content observed when yoghurt bacteria were co-cultured in RSM together. Ammonia was notably produced at a higher level when compared to that of uninoculated RSM. Glutamic acid was utilized to some extent by the combination of *S. thermophilus* 2014 and *L. delbrueckii* ssp. *bulgaricus* 2515 (Table 7.2). There does not appear to be a trend for amino acid content when yoghurt bacteria are in co-culture. However, when probiotic bacteria were incubated together, various amino acids were assimilated by the bacterial cultures. Table 7.2 shows that aspartic acid, threonine, serine, glutamic acid, proline, glycine, alanine, valine, methionine, isoleucine, and leucine were found in lower quantities in probiotic co-cultures when compared to that of uninoculated milk. There did not seem to be a specific preference for any particular amino acid, but the probiotic cultures did assimilate and utilize the amino acids present in the RSM medium.

On the other hand, when yoghurt bacteria were co-cultured with probiotic bacteria, it was observed that the combination of *S. thermophilus* 2002, *L. delbrueckii* ssp. *bulgaricus* 2501, *L. acidophilus* 2415, and *B. thermophilum* 20210 utilized many of the amino acids present in the RSM medium (Table 7.3). To be specific, sulfur-containing amino acids such as methionine and cysteine were very low in that particular combination (0.06 and 0.07 µg/ml respectively) (Table 7.3). The overall total amino acid content of that bacterial combination was much lower than that found in uninoculated RSM, which could indicate that there was competition for the nutrients available between the bacterial cultures. The combination of *S. thermophilum* 2014, *L. delbrueckii* ssp. *bulgaricus* 2515,

L. acidophilus 2405, and *B. longum* 1941 did utilize some amino acids and ammonia was produced. This could be due to the slow growing nature of this combination, in particular *B. longum* 1941.

7.3.2 Amino acid supplementation studies in yoghurt and probiotic bacteria

Cultures were grown in the presence of amino acids, in particular, those containing sulphur, e.g. cysteine and methionine to assess their influence on cell growth and acid production. Sulphur-containing amino acids were examined because it has been reported that cow's' milk was deficient in these amino acids and L-cysteine seems to be the only growth-limiting sulphur-containing amino acid for growth of bifidobacteria (Hassinen *et al.*, 1951 and Dave & Shah, 1998).

Bacterial cultures of *S. thermophilus* 2002, *L. delbrueckii* ssp. *bulgaricus* 2501, *L. acidophilus* 2405, and *B. thermophilum* 20210, were grown alone in single cultures and in co-culture with each other. Control cultures were not supplemented with amino acids. Table 7.4 shows the cell growth and acid production exhibited by *S. thermophilus* 2002 and *L. delbrueckii* ssp. *bulgaricus* 2501 when supplemented with various amino acids in single cultures at 37°C for 24 hours. Table 7.5 shows the cell growth and acid production exhibited by *L. acidophilus* 2405 and *B. thermophilum* 20210 when supplemented with various amino acids in single cultures at 37°C for 24 hours. Table 7.6 shows the cell growth and acid production exhibited by *S. thermophilus* 2002 and *L. delbrueckii* ssp. *bulgaricus* 2501 when supplemented with various amino acids in co-culture at 37°C for 24 hours. Table 7.7 shows the cell growth and acid production exhibited by *L. acidophilus* 2405 and *B. thermophilum*. 20210 when supplemented with various amino acids in co-culture at 37°C for 24 hours.

At 24 hour, growth and acid production of *S. thermophilus* 2002 was in the following order: aspartic acid > threonine > glutamic acid > cysteine + methionine > methionine > cysteine (Table 7.4). The differences in the log cell counts among the treatment groups were significantly lower ($P<0.049$) when compared to the control group (*S. thermophilus*

2002 incubated at 37°C without the supplementation of amino acids). The same observation can be noted for *S. thermophilus* 2002 when incubated in co-culture with the other bacterial organisms (Table 7.6), where its growth and acid production was reduced by the various amino acids in the order: threonine > aspartic acid > cysteine > glutamic acid > cysteine + methionine > methionine (Table 7.6).

Stimulation of growth and acid production of the other yoghurt bacteria, *L. delbrueckii* ssp. *bulgaricus* 2501 by individual amino acids was in the following order: threonine > cysteine > cysteine and methionine > aspartic acid > glutamic acid > methionine (Table 7.4). The differences in the log cell counts among all amino acids, except methionine were significantly higher ($P<0.046$) when compared to the control group (*L. delbrueckii* ssp. *bulgaricus* 2501 incubated at 37°C without the supplementation of amino acids).

However, when *L. delbrueckii* ssp. *bulgaricus* 2501 was in co-culture with *S. thermophilus* 2002, *L. acidophilus* 2504 and *B. thermophilum* 20210, and supplemented with various amino acids, the order of preference for the various amino acids was notably different when compared to that of *L. delbrueckii* ssp. *bulgaricus* 2501 cultured alone. Growth and acid production of *L. delbrueckii* ssp. *bulgaricus* 2501 when mixed with *S. thermophilus* 2002, *L. acidophilus* 2504 and *B. thermophilum* 20210 was in the following order: cysteine > methionine > threonine > glutamic acid > aspartic acid > cysteine and methionine (Table 7.6). When co-cultured with *S. thermophilus* 2002, *L. acidophilus* 2504 and *B. thermophilum* 20210, *L. delbrueckii* ssp. *bulgaricus* 2501 seemed to have a preference for sulfur-containing amino acids, however, the viable log counts were significantly lower ($P<0.01$) among the different treatment groups.

On the other hand, the effect of the supplementation of various amino acids on the bacterial cell counts of the probiotic bacteria, *L. acidophilus* 2405 and *B. thermophilum* 20210 was notably different to that of the yoghurt bacteria studied. More specifically, the growth and acid production of *L. acidophilus* 2405 by individual amino acids was in the following order: glutamic acid > cysteine > cysteine and methionine > methionine > aspartic acid > threonine (Table 7.5). The difference among the treatment groups were

not statistically significant ($P<0.053$) when compared to that of the control *L. acidophilus* 2405 (cultured without the supplementation of amino acids). The amino acid threonine had the weakest influence (4.74 $\log_{10}$) on the viable counts of *L. acidophilus* 2405. It was also observed that *L. acidophilus* 2405 culture had a preference for the sulfur-containing amino acids cysteine, methionine and the combination of cysteine and methionine (Table 7.5). This is in agreement with other research findings where Mikhlin and Radina (1981) stated that the absence of L-cysteine negatively affects bacterial development. A key role for this particular amino acid is attributed to the disulphide bonds and sulphydrilic groups (Sasago *et al.*, 1963; Mikhlin and Radina, 1981) acting as one of the main redox potential donors in milk. On the other hand, significant differences were observed when *L. acidophilus* 2405 was co-cultured with *S. thermophilus* 2002, *L. delbrueckii* ssp. *bulgaricus* 2501, and *B. thermophilum* 20210 and when supplemented with methionine and glutamic acid ($P<0.20$). Growth and acid production by *L. acidophilus* 2405 when in co-culture by individual amino acids was in the following order: methionine > cysteine > cysteine and methionine > glutamic acid > threonine > cysteine > aspartic acid (Table 7.7). These results were significantly different ($P<0.20$) to those observed when *L. acidophilus* 2405 was cultured alone. This could be due to the various interactions that could be arising as a result of the co-culture of these bacterial strains. Examples of such interactions could include the rate of growth of bacterial cultures involved, competition for nutrients, and compounds released by the bacterial strains that could be inhibiting the other bacterial cultures in the mixed culture. The order of preference for the amino acids is different; however, *L. acidophilus* 2405 still preferred the di-sulfide containing amino acids.

Lastly, the influence of supplementation of cysteine, methionine, aspartic acid, cysteine and methionine, glutamic acid and threonine on the cell growth of *B. thermophilum* 20210 when cultured alone was also investigated. The various amino acid supplementations did not significantly influence the log viable counts and acid production of *B. thermophilum* 20210 when compared to that of control *B. thermophilum* 20210 ($P>0.063$).

Growth and acid production of *B. thermophilum*. 20210 by individual amino acids were in the following order: methionine > glutamic acid > aspartic acid > cysteine and methionine > cysteine > threonine (Table 7.5). Bacterial log counts achieved for each treatment group were not significantly different where there was only a 1 log difference. On the other hand, the log viable counts of *B. thermophilum* 20210, when co-cultured with *S. thermophilus* 2002, *L. delbrueckii* ssp. *bulgaricus* 2501, and *L. acidophilus* 2405 was significantly influenced (P>0.20) by methionine, glutamic acid, threonine, and cysteine and methionine supplementations. Stimulation of growth and acid production of *B. thermophilum* 20210, when in co-culture, was in the following order: threonine > methionine > cysteine and methionine > glutamic acid > cysteine > aspartic acid (Table 7.7).

The results showed that *B. thermophilum* 20210 had a preference for the di-sulfide containing amino acids. These results confirmed earlier studies by Cheng and Nagasawa (1983), Collins & Hall (1984) and Roy *et al.* (1990), who observed that cysteine had a stimulatory effect upon growth and acid production by Bifidobacteria cultured in milk. Moreover, Murad *et al.* (1997) found that supplementation of buffalo milk with individual amino acids at a concentration of 3mM lysine, glycine, cysteine and glutamic acid, enhanced growth and acid production of *B. bifidum*. The synergistic effect of the above tested amino acids suggest an important dependence of bifidobacteria species growth upon qualitative and quantitative balance of mixed amino acids and at least contains sulphur containing amino acids (Kamaly, 1997).

7.4 Conclusions

The influence of fortifying milk with cysteine, methionine, aspartic acid, glutamic acid, threonine, and cysteine and methionine on growth and acid production by *S. thermophilus* 2002, *L. delbrueckii* ssp. *bulgaricus* 2501, *L. acidophilus* 2405, and *Bifidobacterium* ssp. 20210 was studied. Total amino acids quantified in the bacterial cultures were less than that of the control RSM, where the lowest was observed for *L. delbrueckii* ssp. *bulgaricus* 2501 and *L. acidophilus* 2405 (39.8 and 43.2 µg/ml respectively). *S. thermophilus* 2002

and *B. thermophilum* 20210 released the highest amounts of tyrosine which indicates a high degree of proteolysis. Both *S. thermophilus* strains (2002 and 2014) had utilized the amino acids aspartic acid, threonine, serine, glutamic acid, alanine, valine, leucine, and lysine. The rest of the amino acids tested were not utilized to a great extent. *L. delbrueckii* ssp. *bulgaricus* 2501 appeared to utilize amino acids to a greater extent than that of *L. delbrueckii* ssp. *bulgaricus* 2515. Total amino acids quantified in *L. delbrueckii* ssp. *bulgaricus* 2515 filtrates (96.4 μg/ml) was much greater than that of *L. delbrueckii* ssp. *bulgaricus* 2501 (39.8 μg/ml). *L. acidophilus* 2405 had a preference for the utilization for most of the amino acids except for tyrosine and cystine and cysteine. On the other hand, *B. thermophilum* 20210 seemed to utilize amino acids more than *B. longum* 1941. When compared to control RSM, *B. thermophilum* 20210 released about 69.9 μg/ml of the total amino acids available compared to 114.6 μg/ml for *B. longum* 1941.

Bacterial cultures were also grown in the presence of amino acids, in particular, those containing sulphur, e.g. cysteine and methionine to assess their influence on cell growth and acid production. Bacterial cultures of *S. thermophilus* 2002, *L. delbrueckii* ssp. *bulgaricus* 2501, *L. acidophilus* 2405, and *B. thermophilum* 20210, were grown alone in single cultures and in co-culture with each other. Control cultures were not supplemented with amino acids. The log cell counts of yoghurt bacteria were significantly different when supplemented with the amino acids compared to the control group (not supplemented with amino acids). The preference for the amino acids tested varied in order from one bacteria to another. On the other hand, the effect of the supplementation with various amino acids on the cell counts of the probiotic bacteria, *L. acidophilus* 2405 and *B. thermophilum* 20210 was notably different to that of the yoghurt bacteria studied

The log viable counts of *B. thermophilum* 20210, when co-cultured with *S. thermophilus* 2002, *L. delbrueckii* ssp. *bulgaricus* 2501, and *L. acidophilus* 2405 was significantly influenced ($P>0.20$) by methionine, glutamic acid, threonine, and cysteine and methionine. The results show that *B. thermophilum* 20210 had a preference for the di-sulfide containing amino acids. The results of these studies show that the probiotic

bacteria had a preference for di-sulfide containing amino acids whereas the yoghurt bacteria had a preference for the other amino acids tested.

Table 7.1 **HPLC quantification of the total amino acids in single bacterial cultures subcultured in 12% RSM at 37°C.**

Amino acid (µg/mL)	Control	ST 2002	ST 2014	LB 2501	LB 2515	LA 2405	LA 2415	BB 20210	BB 1941
Aspartic acid	14.1	2.6	5.9	1.1	6.9	1.2	10.7	2.9	7.1
Threonine	7.1	5.4	3.7	2.8	4.0	2.8	5.7	4.8	4.7
Serine	8.1	4.9	3.8	2.3	4.3	1.6	6.1	4.1	4.5
Glutamic acid	30.6	6.9	9.0	2.1	16.8	2.8	24.7	6.8	19.1
Proline	7.5	2.5	3.4	1.1	4.1	1.2	6.5	1.9	4.8
Glycine	6.0	3.4	3.0	1.6	3.9	1.7	5.2	3.0	4.1
Alanine	8.6	2.8	4.1	1.3	4.4	1.3	6.9	3.3	5.0
Valine	9.7	4.8	4.3	2.5	5.1	2.5	7.2	4.2	5.6
Methionine	2.7	3.6	0.9	2.2	0.8	1.9	1.3	2.5	1.5
Isoleucine	9.0	4.0	4.1	1.9	4.5	1.9	6.7	4.1	5.2
Leucine	12.1	5.2	5.2	2.4	6.1	2.3	9.1	4.8	7.1
Tyrosine	4.7	7.1	2.1	5.5	2.1	6.4	3.2	7.6	2.4
Phenylalanine	6.5	5.2	3.0	2.7	3.2	2.9	4.9	3.8	3.8
Lysine	10.4	1.8	4.9	0.8	5.6	0.8	7.9	2.6	6.6
Histidine	4.0	2.7	1.9	1.4	2.6	1.5	2.8	2.2	2.6
Ammonia	17.0	1.6	15.1	1.0	17.4	0.7	22.7	1.7	24.1
Arginine	5.5	4.3	3.2	2.0	3.8	2.2	5.3	3.5	4.9
Cystine & cysteine	2.8	3.8	0.7	4.7	0.8	7.1	1.0	6.0	1.5
Tryptophan	0.5	0.3	<0.2	0.4	<0.2	0.4	<0.2	0.1	<0.2
Total amino acids	166.9	72.9	78.3	39.8	96.4	43.2	137.9	69.9	114.6

Table 7.2 **HPLC quantification of the total amino acids in mixed cultures subcultured in 12% RSM at 37°C.**

Amino acid (µg/mL)	Control	ST 2002 + LB 2501	ST 2014 + LB 2515	LA 2415 + BB 20210	LA 2405 + BB 1941
Aspartic acid	14.1	13.0	10.2	7.8	8.1
Threonine	7.1	6.9	5.8	4.5	5.5
Serine	8.1	7.9	6.4	4.3	5.4
Glutamic acid	30.6	28.0	13.4	17.7	21.1
Proline	7.5	6.3	5.9	4.4	5.2
Glycine	6.0	6.0	5.2	3.8	4.7
Alanine	8.6	7.7	7.1	5.2	5.7
Valine	9.7	8.3	7.0	5.2	6.5
Methionine	2.7	1.9	1.4	1.2	2.8
Isoleucine	9.0	7.7	6.3	4.7	5.8
Leucine	12.1	10.6	8.3	6.4	7.8
Tyrosine	4.7	3.9	2.4	2.0	2.9
Phenylalanine	6.5	5.7	4.0	3.1	4.3
Lysine	10.4	8.8	7.6	5.8	7.4
Histidine	4.0	4.0	4.7	2.4	2.7
Ammonia	17.0	24.3	23.2	18.0	21.6
Arginine	5.5	5.0	5.2	4.0	4.7
Cystine & cysteine	2.8	1.0	1.2	1.4	2.0
Tryptophan	0.5	<0.2	<0.2	<0.2	<0.2
Total amino acids	166.9	157.0	125.3	101.9	124.2

Table 7.3 **HPLC quantification of the total amino acids in mixed cultures subcultured in 12% RSM at 37°C.**

Amino acid (µg/mL)	Control	ST 2002 + LB 2501 + LA 2415 + BB 20210	ST 2014 + LB 2515 + LA 2405 + BB 1941
Aspartic acid	14.1	7.0	9.6
Threonine	7.1	3.7	5.7
Serine	8.1	3.7	5.4
Glutamic acid	30.6	15.0	14.1
Proline	7.5	3.8	5.8
Glycine	6.0	3.2	4.6
Alanine	8.6	4.2	6.7
Valine	9.7	4.4	6.7
Methionine	2.7	0.6	1.8
Isoleucine	9.0	4.1	6.0
Leucine	12.1	5.6	8.2
Tyrosine	4.7	1.9	2.7
Phenylalanine	6.5	2.8	4.5
Lysine	10.4	4.9	7.4
Histidine	4.0	3.1	2.9
Ammonia	17.0	17.1	25.4
Arginine	5.5	3.5	5.0
Cystine & cysteine	2.8	0.7	1.6
Tryptophan	0.5	<0.2	<0.2
Total amino acids	166.9	89.3	124.1

Table 7.4 Cell growth and acid development (pH) of single cultures of *S. thermophilus* 2002, and *L. delbrueckii* ssp. *bulgaricus* 2501 in milk supplemented with some amino acids and incubated at 37°C for 24h[a]

Additives	Culture			
	S. thermophilus 2002		*L. delbrueckii* ssp. *bulgaricus* 2501	
	Log_{10} cfu/ml	pH[b]	Log_{10} cfu/ml	pH[b]
Milk (control)	8.95 ± 0.01	3.91 ± 0.01	5.82 ± 0.05	3.48 ± 0.05
Milk with added[c]				
Cysteine	6.98 ± 0.02	4.11 ± 0.02	7.13 ± 0.01	3.60 ± 0.01
Methionine	7.93 ± 0.00	3.94 ± 0.00	5.06 ± 0.02	3.46 ± 0.02
Aspartic acid	8.65 ± 0.05	4.03 ± 0.05	6.17 ± 0.01	3.65 ± 0.01
Glutamic acid	8.28 ± 0.01	3.75 ± 0.01	6.00 ± 0.01	3.44 ± 0.01
Threonine	8.63 ± 0.09	3.96 ± 0.09	8.23 ± 0.00	3.45 ± 0.00
Cysteine and methionine	8.20 ± 0.00	3.83 ± 0.00	6.68 ± 0.05	3.36 ± 0.05

[a] Values represent averages of duplicate experiments
[b] Initial pH of RSM and RSM with additives = 6.20-6.30
[c] Each amino acid was added to give final concentration of 50 mg/100 ml and in mixture of amino acids each at 50 mg/100 ml

Table 7.5 Cell growth and acid development (pH) of single cultures of *L. acidophilus* 2405, and *Bifidobacterium* ssp. 20210 in milk supplemented with some amino acids and incubated at 37°C for 24h[a]

Additives	Culture			
	L. acidophilus 2405		*Bifidobacterium* ssp. 20210	
	Log_{10} cfu/ml	pH[b]	Log_{10} cfu/ml	pH[b]
Milk (control)	8.95 ± 0.01	3.77 ± 0.01	9.00 ± 0.00	4.22 ± 0.00
Milk with added[c]				
Cysteine	7.79 ± 0.01	4.10 ± 0.01	8.10 ± 0.05	4.29 ± 0.05
Methionine	7.62 ± 0.17	3.79 ± 0.01	8.80 ± 0.02	4.23 ± 0.02
Aspartic acid	5.80 ± 0.01	4.27 ± 0.01	8.79 ± 0.04	4.22 ± 0.01
Glutamic acid	8.80 ± 0.01	3.89 ± 0.01	8.80 ± 0.01	4.47 ± 0.01
Threonine	4.74 ± 0.04	3.75 ± 0.01	7.92 ± 0.02	4.50 ± 0.01
Cysteine and methionine	7.66 ± 0.06	3.80 ± 0.01	8.22 ± 0.01	4.03 ± 0.00

[a] Values represent averages of duplicate experiments
[b] Initial pH of RSM and RSM with additives = 6.20-6.30
[c] Each amino acid was added to give final concentration of 50 mg/100 ml and in mixture of amino acids each at 50 mg/100 ml

Table7.6 Cell growth of *S. thermophilus* 2002, and *L. delbrueckii* ssp. *bulgaricus* 2501 in co-culture with *L. acidophilus* 2405 and *Bifidobacterium* ssp. 20210 in milk supplemented with some amino acids and incubated at 37°C for 24hour[a]

Additives	Culture	
	***S. thermophilus* 2002**	***L. delbrueckii* ssp. *bulgaricus* 2501**
	Log_{10} cfu/ml	**Log_{10} cfu/ml**
Milk (control)	8.72 ± 0.02	8.45 ± 0.00
Milk with added[b]		
Cysteine	7.72 ± 0.02	7.97 ± 0.02
Methionine	6.78 ± 0.03	7.92 ± 0.04
Aspartic acid	8.03 ± 0.02	7.68 ± 0.04
Glutamic acid	7.04 ± 0.06	7.84 ± 0.06
Threonine	8.58 ± 0.05	7.87 ± 0.48
Cysteine and methionine	6.98 ± 0.07	7.26 ± 0.02

[a] Values represent averages of duplicate experiments

[b] Each amino acid was added to give final concentration of 50 mg/100 ml and in mixture of amino acids each at 50 mg/100 ml

Table 7.7 Cell growth of *L. acidophilus* 2405, and *Bifidobacterium* ssp. 20210 in co-culture with *S. thermophilus* 2002 and *L. delbrueckii* ssp. *bulgaricus* 2501 supplemented with some amino acids and incubated at 37°C for 24hour[a]

Additives	Culture	
	L. acidophilus 2405	*Bifidobacterium* ssp. 20210
	Log_{10} cfu/ml	Log_{10} cfu/ml
Milk (control)	6.77 ± 0.01	6.43 ± 0.03
Milk with added[b]		
Cysteine	6.16 ± 0.01	6.07 ± 0.05
Methionine	7.24 ± 0.01	7.06 ± 0.03
Aspartic acid	4.94 ± 0.01	5.25 ± 0.01
Glutamic acid	7.00 ± 0.00	6.86 ± 0.00
Threonine	6.20 ± 0.02	8.27 ± 0.01
Cysteine and methionine	7.09 ± 0.03	6.95 ± 0.00

[a] Values represent averages of duplicate experiments

[b] Each amino acid was added to give final concentration of 50 mg/100 ml and in mixture of amino acids each at 50 mg/100 ml

Chapter 8 Influence of addition of proteolytic strains of *Lactobacillus delbrueckii* ssp. *bulgaricus* to commercial ABT starter cultures on texture of yoghurt, exopolysaccharide production and survival of bacteria[#]

8.1 Introduction

Many functional characteristics of lactic acid bacteria (LAB) are responsible for their historical and modern use in food production. One such characteristic that has recently generated much interest is polysaccharide production. LAB are capable of producing several different polysaccharides, which can be found in the cytoplasm as carbon and energy sources, as components of the bacterial cell wall, or as external appendages of the cell. Exopolysaccharides (EPS) are polysaccharides external to the cell, either attached to cells or excreted as free polysaccharides (Cerning, 1990; Cerning *et al.*, 1992; Nakajima et al., 1990). Nomenclature for describing EPS isolated from LAB is ambiguous. They have been described as mucoid, slime producing, and ropy, and their compositions and structures are very heterogeneous (Knoshaug *et al.*, 2000).

Ropy EPS-producing LAB strains have been used in Scandinavian countries to produce a thick fermented milk called viili, langfil, or filmjolk (Wacher-Rodarte *et al.*, 1993). Currently, EPS-producing LAB strains are used in yoghurt, sour cream, and whipped toppings to improve their rheological properties, to prevent syneresis, and to replace stabilizers.

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The use of AB (*L. acidophilus* and bifidobacteria) bacteria in yoghurt is increasing due to potential health benefits associated with the consumption of these bacteria. Viability of AB bacteria is of paramount importance in marketing of probiotic products. However, in a previous study (Dave & Shah 1997b), viability of bifidobacteria declined dramatically during manufacture of yoghurt with ABT (*L. acidophilus*, bifidobacteria and *S. thermophilus*) starter culture that produced exopolysaccharides.

Use of exopolysaccharide-producing yoghurt starter cultures is gaining popularity among yoghurt manufacturers (Wacher-Rodarte *et al.*, 1993; Hess *et al.*, 1997). Gassem *et al.* (1995) and Griffin *et al.* (1996) reported that polysaccharide-producing yoghurt bacteria were important determinants of yoghurt viscosity and texture. These starter cultures improve the viscosity of yoghurt leading to resistance to mechanical damage (Tamime & Deeth, 1980). The polysaccharide-producing ABT yoghurt starter cultures have established their market in Australia and Europe.

Hassan *et al.* (1996) studied the textural properties of yoghurt made with encapsulated ropy and non-ropy yoghurt cultures and concluded that the firmness and viscosity were dependent on the pH of yoghurt and the type of starter culture used to manufacture the product. Polysaccharide-producing bacteria are reported to produce yoghurt gels of low fracturability and the apparent viscosity was dependent on the capsule size and bacterial counts (Kailasapathy *et al.*, 1996). Exopolysaccharide cultures of LAB have been recognized for many years as the cause of the viscosity of ropy milk (Escalante *et al.*, 1998). Use of slime-producing strains of *S. thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*, especially in stirred yoghurts, results in a thicker body and higher viscosity, enhanced smoothness, and in prevention of syneresis (Wacher-Rodarte *et al.*, 1993).

In ABT, *S. thermophilus* is the sole fermenting organism. No symbiotic relationship exists during fermentation as *L. delbrueckii* ssp. *bulgaricus* is absent from the starter culture. The three bacteria used in ABT starter culture are reported to be less proteolytic than *L. delbrueckii* ssp. *bulgaricus* (Shihata & Shah, 2000). As a result, the fermentation

is slow with ABT starter cultures and the viability of AB organisms is reported to be low as the organisms require additional nutrients such as amino acids and peptides (Dave & Shah, 1998). Therefore, the addition of proteolytic *L. delbrueckii* ssp. *bulgaricus* to these starter cultures might improve the fermentation process and the viability of AB organisms as the former may release amino acids and peptides through breakdown of protein in milk for the latter.

Therefore, in the present study the effects of addition of proteolytic strains of *L. delbrueckii* ssp. *bulgaricus* to commercial ABT starter cultures on the texture of yoghurt, exopolysaccharide production and viability of probiotic organisms were studied.

8.2 Materials and methods

8.2.1 Bacterial cultures

S. thermophilus-1 (ST-1), *S. thermophilus*-4 (ST-4), *L. acidophilus*-1 (LA-1), *L. acidophilus*-4 (LA-4), bifidobacteria-1 (BB-1) and bifidobacteria-4 (BB-4) were isolated from ABT-1 and ABT-4 commercial starter cultures (Chr. Hansen Pty. Ltd., Bayswater, Australia) using the method described in section 3.2.2 in chapter three. The organisms were propagated separately. *L. delbrueckii* ssp. *bulgaricus* strains 2501 and 2515 were obtained from the Victoria University culture collection (Melbourne, Victoria, Australia) and were selected based on their high proteolytic activity as reported earlier (Shihata & Shah, 2000). Each culture was maintained and propagated according to the method described in section 3.2.1 in Chapter three.

8.2.2 Yoghurt preparation

Yoghurt was prepared according to the method described in section 3.2.10.1 in chapter three.

8.2.3 Time interval specifications

The '0 day' (day 0) analyses were carried out after overnight cold storage of yoghurt samples, and 'week 1', 'week 2', 'week 3' and 'week 4' analyses were carried out after 7, 14, 21 and 28 days of storage, respectively.

8.2.4 *Sample preparation*

Yoghurt prepared in 500 mL cups was used for measuring the firmness of the product at day 0. After measuring the firmness, the yoghurt sample was uniformly mixed and approximately 450 mL sample taken in 500 mL glass beakers for the measurement of viscosity. All the measurements were carried out in triplicate.

8.2.5 Texture analyses

The firmness of the set yoghurt at day 0 was measured with a cone penetrometer (Stanhope-Seta Ltd., Surrey, England) using the method described in section 3.2.10.4 in chapter three.

8.2.6 *Extraction and quantification of exopolysaccharide*

Exopolysaccharide from yoghurts was extracted according to method of Uemura *et al.* (1998) which is explained in section 3.2.10.5 in Chapter three. Total EPS was assayed and the quantification of sugar was carried out using the method of Dubois *et al.* (1956). The amount of EPS was expressed as microgram of glucose equivalents per gram of yoghurt using glucose as a standard.

8.2.7 *Microbiological analyses*

Selective and differential enumeration of yoghurt and probiotic bacteria present in the various yoghurt preparations was carried out according to the method described in section 3.2.2 in Chapter three.

Unless otherwise indicated, all experiments and analyses were replicated 3 times and results presented are averages of the nine replicates.

8.2.8 *Statistical Analysis*

The data was subjected to one-way ANOVA analysis (SigmaStat®, 1997).

8.3 Results and Discussion

The ABT starter culture consists of *L. acidophilus, Bifidobacterium* ssp. and *S. thermophilus*. Previous studies have shown low viability of *Bifidobacterium* ssp. and longer fermentation times for yoghurt production with ABT starter culture (Dave & Shah, 1997a & b). Therefore this study investigated the effects of addition of proteolytic strains of *L. delbrueckii* ssp. *bulgaricus* (Shihata & Shah, 2000) on fermentation time, EPS production, and textural properties of yoghurts and viability of *S. thermophilus, L. delbrueckii* ssp. *bulgaricus, L. acidophilus* and *Bifidobacterium* ssp. The fermentation time and EPS contents of different yoghurts made with various starter culture combinations are presented in Table 8.2.

In general, addition of *L. delbrueckii* ssp. *bulgaricus* 2501 to ABT starter cultures performed better than *L. delbrueckii* ssp. *bulgaricus* 2515 in reducing fermentation time by 50% when compared to ABT-1 alone. The fermentation times with ABT-1 and ABT-4 starter cultures were 10 h and 7 min and 6 h, respectively. Yoghurts made with ABT-1 and ABT-4 starter cultures with added *L. delbrueckii* ssp. *bulgaricus* 2501 fermented in the shortest time of 5 h and 33 min and 5 h and 8 min, respectively. The yoghurts made with the starter cultures ABT-1 and ABT-4 with added *L. delbrueckii* ssp. *bulgaricus* 2515, respectively, were the third quickest to ferment in 6 h and 25 min. The mix starter culture (Table 8.2) with the added *L. delbrueckii* ssp. *bulgaricus* strains (2515 and 2501) showed fermentation times of 6 h, 47 min and 7 h, respectively. The slowest fermentation of 12 h was with the mix starter bacteria, which consisted of *S. thermophilus* 2002, *L. acidophilus* 2415, and bifidobacteria 20210. Thus it appears that addition of proteolytic strain of *L. delbrueckii* ssp. *bulgaricus* helped reduce fermentation time, possibly due to liberation of peptides and amino acids required by less proteolytic bacteria.

The strains of *S. thermophilus* in ABT-1 starter culture and ABT-4 starter culture are different (Dave & Shah, 1997b). *S. thermophilus* in ABT-4 starter culture is used for fast set and ABT-1 is for slow fermentation and *S. thermophilus* strain in ABT-4 starter culture may be more proteolytic than that in ABT-1 starter culture (Chr. Hansen, personal

communication). This could be the reason why there was no major change in the fermentation time when proteolytic strains of *L. delbrueckii* ssp. *bulgaricus* were added to ABT-4 starter culture. The fermentation time decreased by 4 h and 34 min with ABT-1 starter culture supplemented with *L. delbrueckii* ssp. *bulgaricus* 2501 as compared with a drop in the fermentation time of 52 min only with ABT-4 starter culture with the same strain of *L. delbrueckii* ssp. *bulgaricus*. *S. thermophilus* strains are used in combination with *L. delbrueckii* ssp. *bulgaricus* strains as commercial yoghurt starters. However, in ABT starter culture, *S. thermophilus* is the sole fermenting organism. In yoghurt making, *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* grow symbiotically producing essential amino acids for each other and the fermentation time is approximately 4 h. However, no symbiotic relationship exists during yoghurt making, as *L. delbrueckii* ssp. *bulgaricus* is absent from the starter culture. As a result, the fermentation is slow with ABT starter cultures. Supplementation with amino acid and peptides is reported to improve the fermentation process with ABT starter culture and, also, viability of AB organisms (Dave & Shah, 1997b; 1998).

8.3.1 EPS quantification

EPS produced in yoghurt by the different yoghurt starter cultures was extracted with NaOH, precipitated with ethanol, followed by treatments with nuclease and proteinase. The quantity in gram of EPS per 100 gram of yoghurt is shown in Table 8.2. The various mean values of EPS produced among the yoghurts made were not statistically different ($P<0.222$). In general, the quantities of EPS produced by these strains were higher when compared to literature values. Amounts of EPS from *S. thermophilus* can range from as little as 30 mg/L (Cerning *et al.*, 1988) to 890 mg/L (Escalante *et al.*, 1998), and 60 mg/L to 150 mg/L for *L. delbrueckii* ssp. *bulgaricus* strains (Cerning, 1995; Cerning, Bouillanne *et al.*, 1986). This may be due to the associative growth of the starter cultures used in this study, where all three bacterial cultures, *S. thermophilus*, *L. delbrueckii* ssp. *bulgaricus* and *Bifidobacterium* ssp. were exopolysaccharide producers. Bouzar *et al.* (1997) also reported faster EPS production when mixed cultures were used. The yield of EPS produced by LAB also depends on the composition of the medium (carbon and

nitrogen sources) and the conditions in which the organisms are grown (De Vuyst & Degeest, 1999).

8.3.2 Texture analyses

8.3.2.1 Firmness

The firmness of yoghurt (measured as depth of penetration in millimeter) supplemented with *L. delbrueckii* ssp. *bulgaricus* strains 2501 and 2515 are shown in Fig. 8.1. In general, the firmness increased with incorporation of *L. delbrueckii* ssp. *bulgaricus* 2501 to ABT-1 and ABT-4 starter cultures. Yoghurts made with incorporation of *L. delbrueckii* ssp. *bulgaricus* 2501 to ABT-1 or ABT-4 starter cultures were firmer than those with *L. delbrueckii* ssp. *bulgaricus* 2515. The firmness of yoghurts made with ABT-1 starter culture was the lowest ($P<0.001$). Addition of *L. delbrueckii* ssp. *bulgaricus* 2515 to mix culture resulted in a significant ($P<0.001$) firm yoghurt. The depth of cone penetration was 314.7 mm and 374.7 mm, for mix culture with added *L. delbrueckii* ssp. *bulgaricus* 2515 and mix culture with added *L. delbrueckii* ssp. *bulgaricus* 2501, respectively, as compared to 431.7 mm for the yoghurt made with the mix starter culture only. The improvement in firmness with addition of *L. delbrueckii* ssp. *bulgaricus* to the mix culture was better ($P<0.001$) than that with ABT starter cultures. Improvement in firmness, in general, is due to the attachment of mucogenic strains to the protein matrix via the EPS (Marshall & Rawson, 1999).

8.3.2.2 Apparent viscosity

Apparent viscosity of yoghurt samples during refrigerated storage at day "0" is shown in Fig. 8.2. Significant differences ($P<0.001$) were observed in the viscosity of yoghurts supplemented with different combinations of starter cultures. The most viscous yoghurt produced was with mix starter cultures. The addition of *L. delbrueckii* ssp. *bulgaricus* 2501 or 2515 to ABT-1 or to the mix starter culture decreased the viscosity of yoghurt. *L. delbrueckii* ssp. *bulgaricus* 2501 and 2515 are reported to be proteolytic strains (Shihata & Shah, 2000). Proteolytic strains of *L. delbrueckii* ssp. *bulgaricus* are capable of hydrolyzing proteins and this may have led to the reduction in viscosity of yoghurts. There appeared to be a relationship between the fermentation time and viscosity of

yoghurt; as fermentation time increased, the viscosity increased proportionally. Upon addition of proteolytic strains of *L. delbrueckii* ssp. *bulgaricus*, the viscosity decreased. This suggests that the fermentation time could be reduced (Table 8.2) by incorporating proteolytic strains of *L. delbrueckii* ssp. *bulgaricus*, however, this may have adverse effects on viscosity of the product.

The amounts of EPS isolated from yoghurt made with various cultures (Table 8.2) did not correlate with the viscosities of the products. This agrees with previous studies, which stated that the apparent viscosity increased with increasing ropiness, was not simply related to the EPS concentration (Faber *et al.*, 1998). Furthermore, Wacher-Rodarte *et al.* (1993) could not detect any statistically significant correlation between EPS-production and viscosity. However, Cerning *et al.* (1986, 1988) reported a relationship between viscosity and the amount of EPS produced, although this relationship was not always clear.

The quantities of polymer formed by ropy strains of bacterial cultures vary considerably even under identical experimental conditions (Cerning *et al.*, 1990). It is difficult to establish a good correlation between the quantity of polysaccharide produced and the corresponding viscosity. This difficulty may be due to changes in the three-dimensional configuration of polymers and to their interactions with some milk proteins, mainly caseins that are precipitated at low pH. In addition, viscosity measurements are difficult to interpret for non-Newtonian solutions such as milk and fermented milk products (Zourari *et al.*, 1992).

8.3.3 Microbiological analyses

The changes in viable counts of *S. thermophilus*, *L. delbrueckii* ssp. *bulgaricus*, *L. acidophilus*, and *Bifidobacterium* ssp. in yoghurts made with ABT-1 starter cultures supplemented with *L. delbrueckii* ssp. *bulgaricus* 2501 or 2515 over a four week storage period are shown in Table 8.3. There was almost a 2 log increase in the population of *S. thermophilus* during fermentation. *S. thermophilus* in all yoghurt cultures was stable over the 4 week storage period. *L. delbrueckii* ssp. *bulgaricus*, *L. acidophilus*, and

Bifidobacterium ssp. showed a decline in their numbers by week 4. The latter three organisms were able to show a relatively high viable count after 24 h but their numbers declined during storage. *L. acidophilus* in ABT-1 showed the lowest viable count. The addition of *L. delbrueckii* ssp. *bulgaricus* strains (2501 and 2515) improved the viability of probiotic bacteria (*L. acidophilus* and bifidobacteria) when compared with the control yoghurt culture (ABT-1). Table 8.4 shows changes in viable counts of *S. thermophilus*, *L. delbrueckii* ssp. *bulgaricus*, *L. acidophilus*, and *Bifidobacterium* ssp. in ABT-4 starter culture based yoghurts over a four week storage period. There was no improvement in the counts of probiotic bacteria (*L. acidophilus* or bifidobacteria); however, the counts of bifidobacteria were approximately 3 log cycles higher than those of *L. acidophilus* at the end of storage. Table 8.5 shows changes in viable counts of *S. thermophilus*, *L. delbrueckii* ssp. *bulgaricus*, *L. acidophilus*, and *Bifidobacterium* ssp. in mix starter culture based yoghurts over a four week storage period. There was no positive effect of incorporation of *L. delbrueckii* ssp. *bulgaricus* on viability of probiotic bacteria. However, the viability of probiotic bacteria on supplementation with *L. delbrueckii* ssp. *bulgaricus* was the best in yoghurt made with the mix starter culture (Table 8.5) followed by that with ABT-4 (Table 8.4) and ABT-1 (Table 8.3) starter cultures. Dave and Shah (1997a) have shown that the probiotic organisms survived better in ABY (*L. acidophilus*, bifidobacteria, and yoghurt cultures) starter cultures, which contain *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus* than in ABT (*L. acidophilus*, bifidobacteria and *S. thermophilus*) starter cultures. In the present investigation, ABT-4 (Table 8.4) showed better viability of probiotics than ABT-1 starter culture (Table 8.3). This is in agreement with the previous study of Dave and Shah (1997b). ABY starter cultures contain yoghurt bacteria, in particular *L. delbrueckii* ssp. *bulgaricus*, which is more proteolytic than *S. thermophilus*. Symbiotic growth also occurs due to the presence of both bacterial species where they can produce amino acids and peptides, which are required for growth and survival of probiotic bacteria. Supplementation with amino acids and peptides through acid casein hydrolysates has also shown to improve the survival of probiotic organisms (Dave & Shah, 1998).

8.4 Conclusions

Supplementation of proteolytic strain of *L. delbrueckii* ssp. *bulgaricus* to ABT starter cultures reduced fermentation time for making yoghurt and increased firmness. All starter culture combinations examined produced EPS but differences in viscosity did not correlate with the amount of EPS produced. There were no significant differences in EPS production between the cultures studied suggesting that the use of proteolytic strain of *L. delbrueckii* ssp. *bulgaricus* did not influence EPS production. Viability of probiotic bacteria was best in the yoghurt made with mix starter culture with the added *L. delbrueckii* ssp. *bulgaricus* followed by that with ABT-4 and ABT-1 starter cultures.

Table 8.1 Combination of starter cultures used in yoghurt preparations.

Starter culture used for yoghurt production	Bacterial species
ABT[a]-1	ST[b]-1, LA[c]-1, and BB[d]-1
ABT-1 + LB[e] 2501	ST-1, LA-1, BB-1, and LB 2501
ABT-1 + LB 2515	ST-1, LA-1, BB-1, and LB 2515
ABT-4	ST-4, LA-4, and BB-4
ABT-4 + LB 2501	ST-4, LA-4, BB-4, and LB 2501
ABT-4 + LB 2515	ST-4, LA-4, BB-4, and LB 2515
Mix starter culture	ST 2002, LA 2415, and BB 20210
Mix starter culture + LB 2501	ST 2002, LA 2415, BB 20210 and LB 2501
Mix starter culture + LB 2515	ST 2002, LA 2415, BB 20210 and LB 2515

[a] ABT = *L. acidophilus*, and *Bifidobacterium* ssp. and *S. thermophilus.*
[b] ST = *S. thermophilus*
[c] LA = *L. acidophilus*
[d] BB = *Bifidobacterium* ssp.
[e] LB = *L. delbrueckii* ssp. *bulgaricus*

Table 8.2 Fermentation times for preparation of yoghurt to reach pH 4.5, and EPS contents of various types of yoghurts.

Yoghurt sample	Fermentation Times[d]	EPS (g. 100 g^{-1})
ABT[a]-1	10 h 7 min	3.01 ± 0.36
ABT-1 + LB 2501	5 h 33 min	3.41 ± 0.34
ABT-1 + LB 2515	6 h 25 min	3.61 ± 0.45
ABT-4	6 h 0 min	2.76 ± 0.36
ABT-4 + LB[b]2501	5 h 8 min	3.27 ± 0.31
ABT-4 + LB 2515	6 h 25 min	3.56 ± 0.12
Mix starter culture[c]	12 h 0 min	3.01 ± 0.08
Mix + LB 2501	7 h 0 min	2.88 ± 0.19
Mix + LB 2515	6 h 47 min	3.64 ± 0.20

[a] ABT = *L. acidophilus*, and *Bifidobacterium* ssp. and *S. thermophilus.*
[b] LB = *L. delbrueckii* ssp. *bulgaricus*
[c] Mix starter culture = *S. thermophilus* 2002, *L. acidophilus* 2415 and *Bifidobacterium* 20210
[d] Fermentation time was measured by recording initial incubation time till the pH dropped to 4.5

Table 8.3 Changes in viable counts of *S. thermophilus*, *L. delbrueckii* ssp. *bulgaricus*, *L. acidophilus*, and *Bifidobacterium* ssp. in ABT-1 starter cultures during manufacture and storage of yoghurt (ABT = *L. acidophilus*, *Bifidobacterium* ssp. and *S. thermophilus*; ST = *S. thermophilus*; LA = *L. acidophilus;* BB = *Bifidobacterium* ssp.; LB = *L. delbrueckii* ssp. *bulgaricus*).

	0 h	**24 h**	**Week 1**	**Week 2**	**Week 3**	**Week 4**
ST-1	6.93 ± 0.02	8.95 ± 0.06	9.08 ± 0.02	8.93 ± 0.03	9.25± 0.02	9.13± 0.02
LA-1	4.20 ± 0.01	6.78 ± 0.00	6.00 ± 0.02	3.91 ± 0.00	3.69 ± 0.04	2.59 ± 0.03
BB-1	4.10 ± 0.05	5.89 ± 0.01	5.81 ± 0.00	4.64 ± 0.04	4.58 ± 0.01	2.44 ± 0.04
ST-1[1] (ABT-1+LB 2501)	6.92 ± 0.02	9.12 ± 0.01	9.31 ± 0.01	9.11 ± 0.01	9.18 ± 0.01	9.25 ± 0.03
LB 2501	4.81 ± 0.00	8.01 ± 0.02	6.88 ± 0.00	6.34 ± 0.02	6.15 ± 0.04	4.63 ± 0.03
LA-1[2] (ABT-1+LB 2501)	4.27 ± 0.01	6.19 ± 0.01	4.29 ± 0.02	3.99 ± 0.01	3.91 ± 0.01	3.81 ± 0.03
BB-1[3] (ABT-1+LB 2501)	4.10 ± 0.01	5.00 ± 0.01	6.08 ± 0.04	3.68 ± 0.08	3.54 ± 0.08	3.48 ± 0.02
ST-1[4] (ABT-1+LB 2515)	6.93 ± 0.01	9.20 ± 0.00	9.27 ± 0.04	9.10 ± 0.00	9.22 ± 0.04	9.21 ± 0.04
LB 2515	4.57 ± 0.04	7.75 ± 0.03	7.71 ± 0.01	7.10 ± 0.01	6.13 ± 0.00	5.14 ± 0.02
LA-1[5] (ABT-1+ LB 2515)	4.20 ± 0.08	5.94 ± 0.02	4.05 ± 0.01	3.94 ± 0.03	3.85 ± 0.01	3.72 ± 0.01
BB-1[6] (ABT-1+LB 2515)	4.08 ± 0.01	5.17 ± 0.03	3.89 ± 0.01	3.66 ± 0.03	3.87 ± 0.05	2.73 ±0.02

[1]ST counts in yoghurt made with ABT-1 starter culture and LB 2501; [2]LA counts in yoghurt made with ABT-1 starter culture and LB 2501; [3]BB counts in yoghurt made with ABT-1 starter culture and LB 2501; [4]ST counts in yoghurt made with ABT-1 starter culture and LB 2515; [5]LA counts in yoghurt made with ABT-1 starter culture and LB 2515; [6]BB counts in yoghurt made with ABT-1 starter culture and LB 2515.

Table 8.4 Changes in viable counts of *S. thermophilus*, *L. delbrueckii* ssp. *bulgaricus*, *L. acidophilus*, and *Bifidobacterium* ssp. in ABT-4 starter cultures during manufacture and storage of yoghurt (ABT = *L. acidophilus*, *Bifidobacterium* ssp. and *S. thermophilus*; ST = *S. thermophilus*; LA = *L. acidophilus;* BB = *Bifidobacterium* ssp.; LB = *L. delbrueckii* ssp. *bulgaricus*).

	0 h	**24 h**	**Week 1**	**Week 2**	**Week 3**	**Week 4**
ST-4	6.04 ± 0.04	9.00 ± 0.03	8.88 ± 0.03	9.00 ± 0.03	9.88 ± 0.08	8.84 ± 0.06
LA-4	5.95 ± 0.00	7.17 ± 0.01	7.20 ± 0.02	6.66 ± 0.06	4.87 ± 0.01	4.25 ± 0.01
BB-4	6.00 ± 0.01	6.99 ± 0.03	6.85 ± 0.03	6.62 ± 0.01	6.80 ± 0.04	6.69 ± 0.04
ST-4[1] (ABT-4+LB 2501)	6.08 ± 0.03	9.21 ± 0.01	9.16 ± 0.00	9.96 ± 0.10	9.45 ± 0.48	8.90 ± 0.02
LB 2501	5.99 ± 0.02	8.10 ± 0.06	8.07 ± 0.03	7.81 ± 0.03	7.34 ± 0.01	7.06 ± 0.02
LA-4[2] (ABT-4+LB2501)	5.97 ± 0.01	7.18 ± 0.01	5.91 ± 0.03	4.27 ± 0.09	3.67 ± 0.04	3.17 ± 0.02
BB-4[3] (ABT-4+LB2501)	6.02 ± 0.02	6.94 ± 0.04	6.58 ± 0.02	6.54 ± 0.03	6.66 ± 0.08	6.70 ± 0.02
ST-4[4] (ABT-4+LB 2515)	6.15 ± 0.02	8.99 ± 0.02	9.36 ± 0.61	8.90 ± 0.00	8.99 ± 0.02	8.91 ± 0.01
LB 2515	5.99 ± 0.02	7.47 ± 0.01	7.06 ± 0.03	6.29 ± 0.01	6.22 ± 0.02	7.11 ± 0.03
LA-4[5] (ABT-4+LB2515)	5.95 ± 0.01	7.18 ± 0.02	7.20 ± 0.02	5.09 ± 0.01	3.91 ± 0.03	3.88 ± 0.03
BB-4[6] (ABT-4+LB2515)	5.97 ± 0.02	6.72 ± 0.01	6.83 ± 0.04	6.86 ± 0.04	6.68 ± 0.02	6.68 ± 0.04

[1]ST counts in yoghurt made with ABT-4 starter culture and LB 2501; [2]LA counts in yoghurt made with ABT-4 starter culture and LB 2501; [3]BB counts in yoghurt made with ABT-4 starter culture and LB 2501; [4]ST counts in yoghurt made with ABT-4 starter culture and LB 2515; [5]LA counts in yoghurt made with ABT-4 starter culture and LB 2515; [6]BB counts in yoghurt made with ABT-4 starter culture and LB 2515.

Table 8.5 Changes in viable counts of *S. thermophilus*, *L. delbrueckii* ssp. *bulgaricus*, *L. acidophilus*, and *Bifidobacterium* ssp. in mix starter cultures during manufacture and storage of yoghurt (Mix starter culture = ST 2002, LA 2415, BB 20210 and LB 2501; ST = *S. thermophilus*; LA = *L. acidophilus;* BB = *Bifidobacterium* ssp.; LB = *L. delbrueckii* ssp. *bulgaricus*).

	0 h	**24 h**	**Week 1**	**Week 2**	**Week 3**	**Week 4**
ST-Mix	4.98 ± 0.03	7.84 ± 0.09	7.93 ± 0.01	8.95 ± 0.00	7.69 ± 0.04	7.42 ± 0.04
LA-Mix	4.54 ± 0.01	7.69 ± 0.09	7.27 ± 0.01	7.28 ± 0.01	7.07 ± 0.01	7.19 ± 0.01
BB-Mix	4.68 ± 0.03	7.05 ± 0.06	7.09 ± 0.03	7.23 ± 0.01	7.73 ± 0.03	6.72 ± 0.04
ST[1] (Mix + LB 2501)	4.92 ± 0.08	7.65 ± 0.11	8.19 ± 0.01	9.12 ± 0.01	7.95 ± 0.01	7.96 ± 0.01
LB 2501	5.97 ± 0.02	7.85 ± 0.04	7.88 ± 0.02	7.87 ± 0.02	7.56 ± 0.04	6.92 ± 0.00
LA[2] (Mix + LB 2501)	4.52 ± 0.01	6.92 ± 0.02	6.94 ± 0.01	6.90 ± 0.01	6.82 ± 0.03	6.92 ± 0.03
BB[3] (Mix + LB 2501)	4.70 ± 0.03	6.61 ± 0.02	6.70 ± 0.10	6.66 ± 0.04	7.28 ± 0.01	6.30 ± 0.02
ST[4] (Mix + LB 2515)	4.96 ± 0.01	7.09 ± 0.09	8.20 ± 0.00	7.65 ± 0.08	6.82 ± 0.04	8.23 ± 0.03
LB 2515	6.00 ± 0.01	7.86 ± 0.07	7.96 ± 0.05	7.82 ± 0.05	7.50 ± 0.02	6.94 ± 0.02
LA[5] (Mix + LB 2515)	4.50± 0.02	6.87 ± 0.02	6.80 ± 0.02	6.80 ± 0.02	6.20 ± 0.01	6.94 ± 0.01
BB [6] (Mix + LB 2515)	4.90 ± 0.01	6.68 ± 0.09	6.74 ± 0.03	6.60 ± 0.07	7.29 ± 0.01	6.33 ± 0.03

[1]ST counts in yoghurt made with Mix starter culture and LB 2501; [2]LA counts in yoghurt made with Mix starter culture and LB 2501; [3]BB counts in yoghurt made with Mix starter culture and LB 2501; [4]ST counts in yoghurt made with Mix starter culture and LB 2515; [5]LA counts in yoghurt made with Mix starter culture and LB 2515; [6]BB counts in yoghurt made with Mix starter culture and LB 2515

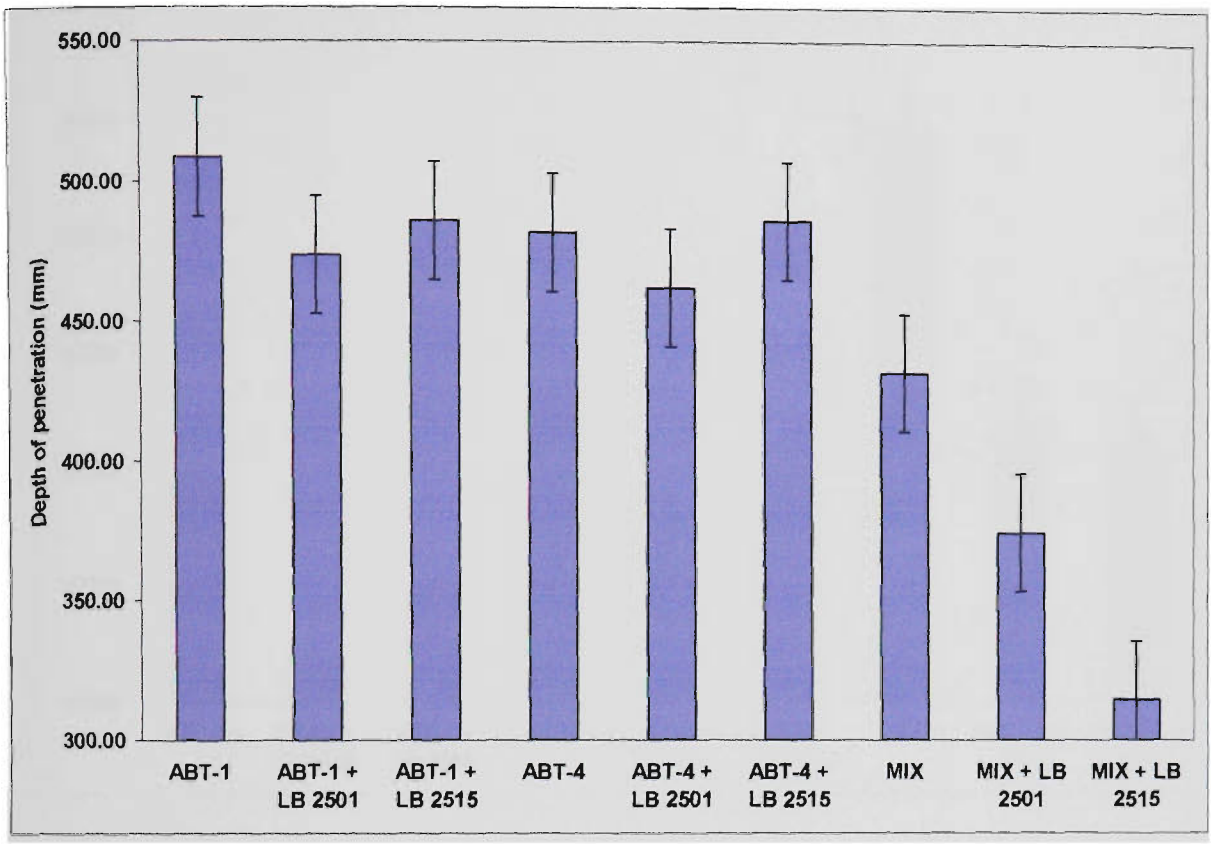

Figure 8.1 Firmness of yoghurt measured as penetration depth (mm) affected by supplementation of *L. delbrueckii* ssp. *bulgaricus* strains (ABT = *L. acidophilus; Bifidobacterium* sp. and *S. thermophilus*; ST = *S. thermophilus*; LA = *L. acidophilus*; BB = *Bifidobacterium* sp.; LB = *L. delbrueckii* ssp. *bulgaricus*).

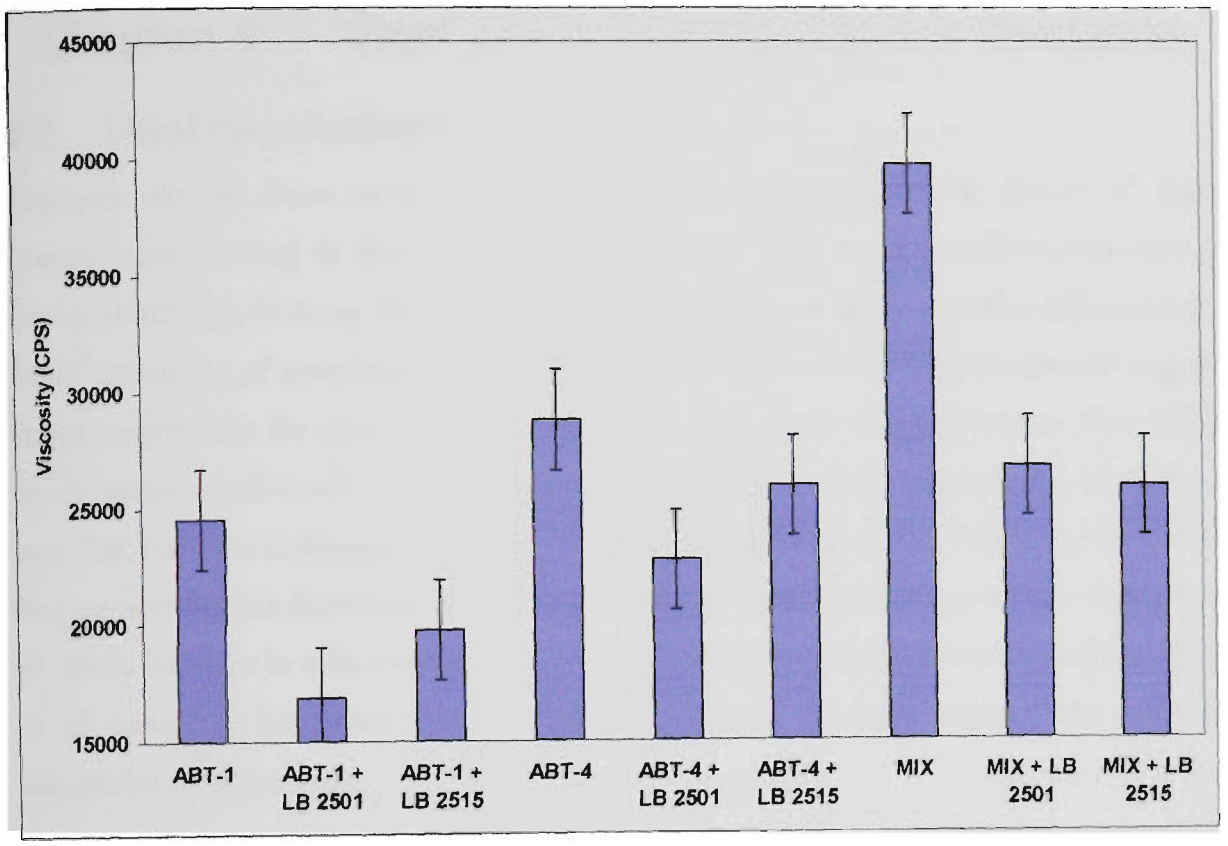

Figure 8.2 **Viscosity of yoghurt samples after supplementation of *L. delbrueckii* ssp. *bulgaricus* strains (ABT = *L. acidophilus; Bifidobacterium* sp. and *S. thermophilus*; ST = *S. thermophilus*; LA = *L. acidophilus*; BB = *Bifidobacterium* sp.; LB = *L. delbrueckii* ssp. *bulgaricus*).**

Chapter 9 Final conclusions and future directions

9.1 Final conclusions

Peptides derived from milk protein hydrolysis are nowadays the object of many investigations owing to their functional properties. The main transformation, due to bacterial activity, is lactic fermentation, and in addition to the production of lactic acid, small quantities of acetylmethylcarbinol, 2, 3- butanedione, acetylaldehyde and organic acids, responsible for aroma, are also detected. This study was carried out after initial investigations had found that certain peptides and amino acids improved the viability of probiotic bacteria in fermented milk products (Dave & Shah, 1998). Thus the aim was to find an appropriate formulation of yoghurt bacteria that would improve the viability of probiotic bacteria in a fermented milk product. This investigation was carried out by an initial screening for proteolytic and peptidase activity for both yoghurt and probiotic bacterial strains provided.

The yoghurt bacteria (*S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus*) appeared to be highly proteolytic as compared to the probiotic bacteria (*L. acidophilus* and *Bifidobacterium* ssp.). The yoghurt bacteria released higher amounts of free amino acids and demonstrated greater aminopeptidase and dipeptidyl activity than the probiotic bacteria. As a result, yoghurt bacteria grow faster in milk, whereas the probiotic bacteria grow slowly due to lack of proteolytic activity and require an exogenous supply of peptides and amino acids for optimum growth, in particular for starter cultures that do not contain *L. delbrueckii* ssp. *bulgaricus*. The associative relationship between various combinations of yoghurt and probiotic bacteria was studied to find the best combination of bacteria that would survive the longest time in a fermented milk product. This study screened for antagonism between yoghurt bacteria and probiotic bacteria which may be responsible for low viability of probiotic bacteria. A total of nine strains of yoghurt bacteria and probiotic bacteria were used in this study. *S. thermophilus* 2002 and both *L. delbrueckii* ssp. *bulgaricus* 2501 and 2515 strains showed strong inhibition due to bacteriocin like inhibitory substance (BLIS) produced by both *L. acidophilus* strains. *Bifidobacterium* ssp. was not inhibited in this study.

Extensive casein degradation and changes in the electrophoretic pattern of the proteins present in the milk fractions were observed during fermentation of cultured milks at 37°C. Proteolysis was faster in milk samples that were fermented by bacterial cultures in single culture or in co-culture with other bacterial cultures and slower in milks fermented with yoghurt and probiotic bacterial strains isolated from dairy starter cultures. All casein fractions incubated with the cell wall and intracellular samples were digested at a faster rate than that in cell wall extracts.

Most bacterial strains showed 0% hydrolysis of α-casein, β-casein, and κ-casein at the cell wall level when compared to that at the intracellular level. *S. thermophilus* 2002 degraded α-casein and β-casein at the fastest rate when compared to other single bacterial cultures studied. Similar results were observed with *S. thermophilus* 2014 except for its action towards κ-casein showing a preference for hydrolysis at the intracellular level. Both *L. delbrueckii* ssp. *bulgaricus* 2501 and 2515 readily attacked all casein fractions, particularly at the intracellular level. Most of the hydrolysis occurred after 4 hours of incubation. *L. acidophilus* (strains 2405 and 2415) also attacked the casein fractions at the intracellular level, where 64%, 70%, and 82% hydrolysis of α-, β-, and κ-casein, occurred, respectively.

In contrast, *B. thermophilum* 20210, and *B. infantis* 1912 did show caseinolytic activity, but slightly a different pattern to that observed with the other bacterial strains tested. *B. infantis* 1912 appeared to show casein hydrolysis at both cell levels; however the rate of hydrolysis was slower than that of the other probiotic strain and yoghurt strains. *B. thermophilum* 20210 grows faster than that of *B. infantis* 1912 where hydrolysis of the casein fractions started as early as after 2 hours of incubation.

The bovine casein group (α, β, and κ-casein) and the whey proteins (β-lactoglobulin and α-lactalbumin) were separated and quantified at the same time using reverse-phase HPLC. *S. thermophilus* 2014 and *L. acidophilus* 2405 showed a preference for α-casein degradation when compared to the other single bacterial cultures studied. On the other hand, *Bifidobacterium* ssp. (1941 and 20099) appeared to hydrolyse α-casein at a slower

rate. *B. thermophilum* (20210) appeared to break the α-casein fraction into a smaller peptide, which indicates that it actively degraded the casein fraction at a faster rate than the other *Bifidobacterium* ssp. strains studied. It appears that the combination of yoghurt bacteria in a mixed culture did degrade α-casein at a faster rate than when cultured alone as would have been expected, indicating that this mixture actively broke down existing bonds and formed smaller peptide fractions.

The other yoghurt bacteria, *L. delbrueckii* ssp. *bulgaricus* 2501 and 2515 did not appear to degrade β-casein at the same rate as that exhibited by *S. thermophilus* strains. *L. delbrueckii* ssp. *bulgaricus* 2501 was able to break down peptide bonds within the κ-casein fraction of milk proteins. It was observed that all mixed bacterial cultures studied degraded κ-casein to some extent, when compared to that of κ-casein in the control milk. There was no particular trend observed for α-lactalbumin utilisation by the mixed bacterial cultures.

The influence of fortifying milk with some amino acids on growth and acid production by *S. thermophilus* 2002, *L. delbrueckii* ssp. *bulgaricus* 2501, *L. acidophilus* 2405, and *Bifidobacterium* ssp. 20210 was studied. Total amino acids quantified in the bacterial cultures were less than those of the control RSM, where the lowest was observed for *L. delbrueckii* ssp. *bulgaricus* 2501 and *L. acidophilus* 2405 (39.8 and 43.2 μg/ml respectively). *S. thermophilus* 2002 and *B. thermophilum* 20210 released the highest amounts of tyrosine which indicates a high degree of proteolysis. Both *S. thermophilus* strains (2002 and 2014) had utilized the amino acids aspartic acid, threonine, serine, glutamic acid, alanine, valine, leucine, and lysine. The rest of the amino acids tested were not utilized to a great extent. *L. delbrueckii* ssp. *bulgaricus* 2501 appeared to utilize amino acids to a greater extent than that of *L. delbrueckii* ssp. *bulgaricus* 2515. Total amino acids quantified in *L. delbrueckii* ssp. *bulgaricus* 2515 filtrates (39.8 μg/100ml) was much greater than that of *L. delbrueckii* ssp. *bulgaricus* 2501 (96.6 μg/ml). *L. acidophilus* 2405 utilized a greater quantity of amino acids than *L. acidophilus* 2415, 43.2 μg/ml and 138.1 μg/ml, respectively. *L. acidophilus* 2405 had a preference for the utilization for most of the amino acids except for tyrosine and cystine and cysteine. On

the other hand, *B. thermophilum* 20210 appeared to utilize amino acids faster than that of *B. longum* 1941. When compared to control RSM, *B. thermophilum* 20210 utilized about 69.9 µg/ml of the total amino acids available compared to 114.6 µg/ml for *B. longum* 1941.

Bacterial cultures were also grown in the presence of amino acids, in particular, those containing sulphur, e.g. cysteine and methionine to assess their influence on cell growth and acid production. Bacterial cultures of *S. thermophilus* 2002, *L. delbrueckii* ssp. *bulgaricus* 2501, *L. acidophilus* 2405, and *B. thermophilum* 20210, were grown alone in single cultures and in co-culture. Control cultures were not supplemented with amino acids. The differences in the log cell counts among the yoghurt bacteria were significantly different when supplemented with the amino acids when compared to the control group (not supplemented with amino acids). The preference for the amino acids tested varied within the bacterial cultures studied. In contrast, the effect of the supplementation of various amino acids on the bacterial cell counts of the probiotic bacteria, *L. acidophilus* 2405 and *B. thermophilum* 20210 was notably different to that of the yoghurt bacteria studied.

The log viable counts of *B. thermophilum* 20210, when co-cultured with *S. thermophilus* 2002, *L. delbrueckii* ssp. *bulgaricus* 2501, and *L. acidophilus* 2405 was significantly influenced ($P>0.20$) by the various amino acid supplementations. The results of these studies showed that the probiotic bacteria had a preference for di-sulfide containing amino acids whereas the yoghurt bacteria had a preference for the other amino acids tested.

Further studies with a particular starter culture supplied by Chr. Hansen showed that supplementation of a proteolytic strain of *L. delbrueckii* ssp. *bulgaricus* to ABT starter cultures reduced fermentation time for making yoghurt and increased firmness. All starter culture combinations examined produced EPS but differences in viscosity did not correlate with the amount of EPS produced. There were no significant differences in EPS production between the cultures studied suggesting that use of proteolytic strain of *L.*

delbrueckii ssp. *bulgaricus* did not influence EPS production. Viability of probiotic bacteria was best in the yoghurt made with mix starter culture with the added *L. delbrueckii* subsp. *bulgaricus* followed by that with ABT-4 and ABT-1 starter cultures.

9.2 Recommendations for future research studies

Further studies into the influence of fortifying milk with some carbohydrates, protein hydrolysates on growth and acid production of yoghurt and probiotic bacteria could give more insight into the proteolytic nature of these bacteria and their preference for nutritional requirements if any. From the studies conducted in this current project, it appears that the combination of *S. thermophilus*, *L. delbrueckii* ssp. *bulgaricus*, *L. acidophilus*, and *Bifidobacterium* ssp. is a very complicated one where *S. thermophilus* is a strict aerobe and the others are microaerophilic. This makes it difficult when adding external nutrients such as cysteine because this in turn breaks down in the fermentation process providing a redox potential and hence an anaerobic environment thus reduces the viability and growth of *S. thermophilus*. The inclusion of *S. thermophilus* in a yoghurt mix is very vital, especially when in symbiosis with *L. delbrueckii* ssp. *bulgaricus* and the latter is also needed to reduce the acidity in the final yoghurt product in an acceptable fermentation time for the dairy industry. It is proposed that further studies into the effect of the exclusion of *S. thermophilus* from the yoghurt production could be done.

It appears that the mixture of yoghurt and probiotic bacteria used was not a successful one as hypothesized in this study. It was hypothesized the fast growing proteolytic yoghurt bacteria; *S. thermophilus* 2002, *S. thermophilus* 2014, *L. delbrueckii* ssp. *bulgaricus* 2501, and *L. delbrueckii* ssp. *bulgaricus* 2515 would produce peptides and amino acids in abundance to improve the growth rate of the probiotic bacteria (*L. acidophilus* and *Bifidobacterium* ssp.) when in mixture. *S. thermophilus* strains did not survive very well when in co-culture with the other bacterial groups, in particular, when $_{\text{di-sulfide}}$ containing amino acids were added to the milk medium. Reasons attributed to this observation could be that *S. thermophilus* is a strict aerobe and their growth is not supported under the redox potential produced by the $_{\text{di-sulfide}}$ containing amino acids. In contrast, the other bacterial groups survived well under these conditions because they are

strict anaerobes in nature. These observations agree with earlier work of Dave & Shah (1997) where high levels of cysteine (250 or 500 mg/L) affected the growth of *S. thermophilus* and the cell morphology of *S. thermophilus* was adversely affected with increased concentration of cysteine (500 mg/L) as a result of the reduced redox potential. Therefore further studies into the selection of strains needs to be carried out.

It has long been recognized that peptides are of great nutritional value for many types of bacteria and that bacteria usually hydrolyse peptides and use the amino acids either directly for protein synthesis, or after further breakdown, as sources of nitrogen, carbon, or sulphur. On the other hand, there are many factors affecting the peptides transportation into the bacterial cell. A further study into the size of the peptides detected in the bacterial filtrates could give a better understanding to the limitations involved with the process of peptide uptake with regards to size. However, it may be presumed that some of the smaller peptides derived from the decomposition of the larger oligopeptides may be taken up directly into the bifidobacteria and utilized for their growth and acid production.

A better understanding of the nature and localization of extracellular and intracellular peptidases of yoghurt and probiotic bacteria may be considered to play an important role in the utilization of the peptides for their growth when in co-culture with yoghurt bacteria. Previous studies have indicated a relationship between the enzyme aminopeptidase and the stimulation of the growth of *B. breve* in incubated milk with *L. casei* and *B. breve* (Cheng & Nagasawa, 1985). Therefore, if the bacterial strains in this study were to be further investigated for their potential as a suitable dairy starter culture, further research into their peptidase activity would be required. To be specific, the enzymes possessed by these bacterial strains would be required to be isolated, extracted, purified, and characterized, in particular, the aminopeptidase enzyme because of the large role it has with *Bifidobacterium* ssp. The enzymes can be then added to milks fermented with bacteria to examine if they affect the growth rate and survival of the bacteria, in particular, the probiotic bacteria.

Since it has been established that peptides and amino acids play an important role in the stimulation of growth of yoghurt and probiotic bacteria, further investigations would be required, where these peptides could be identified, isolated, extracted, purified, characterized and sequenced then fortified (different concentrations) in milk containing probiotic bacteria to find out if they can stimulate a faster growth and survival rate. Further studies into peptide hydrolysates could benefit this study as it has been shown that these are good stimulants for the growth of *Bifidobacterium* strains.

Further research studies into this investigation could include the isolation and characterization of enzymatic peptidases from the most proteolytic yoghurt and probiotic bacteria studied. Once isolated, their action towards the caseins and whey proteins could be further examined which could be further followed by identification, isolation and characterization of the breakdown products that arise from protein degradation. Protein breakdown products can be isolated, sequenced, extracted, and purified and added to a particular starter culture combination and fermented products to examine their effect on the viability of probiotic bacteria. Further investigations into the nature and characteristics of exopolysaccharides produced by the various yoghurt and probiotic bacteria could also benefit various food industries.

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Chapter 11 Appendix

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Proteolytic profiles of yogurt and probiotic bacteria

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Abstract

Nine strains of *Streptococcus thermophilus*, 6 strains of *Lactobacillus delbrueckii* ssp. *bulgaricus*, 14 strains of *Lactobacillus acidophilus* and 13 strains of *Bifidobacterium* spp. were screened for proteolytic, amino-, di-, tri- and endopeptidase activity by using the *o*-pthaldialdehyde-based spectrophotometric assay. Strains showing the highest and lowest proteolytic activity were further studied for their peptidase activities at the extracellular and intracellular levels. The amounts of free amino groups released by *S. thermophilus*, *L. delbrueckii* ssp. *bulgaricus* and *L. acidophilus* strains were higher than that by *Bifidobacterium* strains. Aminopeptidase activity was detected for all bacterial strains both at the extracellular and intracellular levels. The specific activity towards the six substrates studied was higher at the intracellular level for all strains. High dipeptidase activity was demonstrated by all bacterial strains for *L. delbrueckii* ssp. *bulgaricus*, *L. acidophilus*, and *Bifidobacterium* spp. whereas *S. thermophilus* had greater dipeptidase activity at the extracellular level. All bacterial cultures tested were able to hydrolyse large biologically active peptides, bradykinin, Ala-Ala-Ala-Ala-Ala and the tripeptide substrate Gly-Ala-Tyr at both the extracellular and intracellular levels. However, with the substrates ending with a C-terminal of phenylalanine, the hydrolysis only occurred at the intracellular level.

Keywords: Proteolytic activity; OPA; Probiotic bacteria; Yogurt bacteria

1. Introduction

Probiotic bacteria (*Lactobacillus acidophilus* and *Bifidobacterium* spp.) grow slowly in milk because of lack of proteolytic activity (Klaver, Kingma, & Weerkamp, 1993), and the usual practice is to add yogurt bacteria (*Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*) to probiotic products to reduce the fermentation time. *Lactobacillus delbrueckii* ssp. *bulgaricus* produces essential amino acids owing to its proteolytic nature, and the symbiotic relationship of *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus* is well established; the former organism produces amino nitrogen for the latter organism.

Lactic acid bacteria (LAB) are characterised by their high demand for essential growth factors such as peptides and amino acids. However, milk does not contain sufficient free amino acids and peptides to allow growth of LAB (Zourari, Accolas, & Desmazeaud, 1992; Abu-Tarboush, 1996). Therefore, these LAB possess a complex system of proteinases and peptidases, which enable them to use milk casein as a source of amino acids and nitrogen. The first step in casein degradation is mediated by cell wall located proteases, which cleave casein to oligopeptides. Further degradation to smaller peptides and amino acids that can pass through the cell membrane is performed by peptidases (Wohlrab & Bockelmann, 1992). The proteolytic activities of LAB including yogurt bacteria and probiotic bacteria have been studied extensively and proteolytic enzymes have been isolated and characterised (Booth, Jennings, Ni Fhaolain, & O'Cuinn, 1990; Wohlrab & Bockelmann, 1993; Bockelmann, Hoppe-Seyler, & Heller, 1996; Law & Haandrikman, 1997).

Several endopeptidases have been purified from yogurt bacteria including *L. delbrueckii* ssp. *bulgaricus* B14 (Bockelmann et al., 1996), and *S. thermophilus* CNRZ160

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Abbreviations: EE = cell wall extracellular extract, IE = intracellular extract, MRS = de Mann Rogosa Sharpe, OPA = *o*-phthaldialdehyde, RSM = reconstituted skim milk, TCA = trichloroacetic acid, LAB = lactic acid bacteria.

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smazeaud, 1974). Aminopeptidases are thought to be ltimate importance for the development of flavour in mented milk products, since they are capable of releas- single amino acid residues from oligopeptides formed extracellular proteinase activity. Metal-dependent inopeptidases with a broad range specificity were aracterised from *L. delbrueckii* subsp. *bulgaricus* B14 ockelmann, Beuk, Lick, & Heller, 1995), *L. delbrueckii* bsp. *bulgaricus* (Atlan, Laloi, & Portalier, 1989), *L. idophilus* R-26 (Machuga & Ives, 1984), *S. thermophilus* CA-DC 114 (Tsakalidou & Kalantzopolous, 1992) and *thermophilus* CNRZ 302.

Bifidobacterium spp. is comparable to LAB by the resence of a general aminopeptidase activity encom- assing several dipeptidases and possibly iminopeptidase nd tripeptidases (Bockelmann & Fobker, 1991; Egg- nann & Bachmann, 1980; Meyer & Jordi, 1987). minopeptidase and iminopeptidase activities were iso- ated from a cell-free extract of *B. breve* by Cheng and Vagasawa (1984, 1985). *B. infantis*, *B. longum*, and *B. dolescentis* showed aminopeptidase, dipeptidase, tripep- idase and carboxypeptidase activities (El-Soda, Macedo, & Olson, 1992).

Dipeptidases have been purified from *S. thermophilus* Rabier & Desmazeaud, 1973), and *L. delbrueckii* ssp. *bulgaricus* B14 (Wohlrab & Bockelmann, 1992) and tripeptidases from *L. delbrueckii* subsp. *bulgaricus* B14 (Bockelmann et al., 1995; Argyle, Mathison, & Chandan, 1976) and *S. thermophilus* CNRZ160 (Rabier & Des- mazeaud, 1973).

In recent years, fermented milk products containing *L. acidophilus* and *Bifidobacterium* spp. have been de- veloped. There is an increasing interest in dairy products containing specific bacterial species with potential health benefits (Portier, Boyaka, & Bougoudaga, 1993). How- ever, the slow growth of *L. acidophilus* and *Bifidobac- terium* spp. poses major difficulties for market expansion of probiotic products (Brasheras & Gilliland, 1995). Use of most proteolytic strains of *S. thermophilus*, *L. de- lbrueckii* subsp. *bulgaricus*, *L. acidophilus* and *Bifidobac- terium* spp. could enhance the growth and viability of probiotic bacteria in products over the storage period.

In this report, we present an evaluation of the pro- teolytic ability of various strains of *S. thermophilus*, *L. delbrueckii* ssp. *bulgaricus*, *L. acidophilus* and *Bifidobac- terium* spp. Their peptidase profile including amino-, di-, tri- and endopeptidase activities is also reported.

2. Materials and methods

2.1. Bacterial cultures

Pure cultures of 6 strains of *S. thermophilus*, 5 strains of *L. delbrueckii* ssp. *bulgaricus*, 13 strains of *L. acidophilus* and 12 strains of *Bifidobacterium* spp. (*B. breve*, *B. infan- tis*, *B. adolescentis*, *B. longum*, *B. pseudolongum*, *B. thermo- philum*, *Bifidobacterium* spp., and *B. bifidum*) were ob- tained from the culture collection of Victoria University of Technology (Melbourne, Victoria, Australia). Three strains of *S. thermophilus* (T-1, T-4, Y-1), one strain of *L. delbrueckii* ssp. *bulgaricus* (Y-2), and one strain each of *L. acidophilus* (B-1) and *bifidobacteria* (B-2) were isolated from commercial starter cultures (Chr. Hansen, Pty. Ltd., Bayswater, Australia). Each culture was maintained in 12% sterile reconstituted skim milk (RSM) supplemented with 2% glucose and 1% yeast extract. All cultures were stored at −80°C and working cultures were propagated successively three times before use and stored at 4°C.

2.2. Proteolytic activity in reconstituted skim milk

All the bacterial strains were grown overnight at 37°C in de Mann Rogosa Sharpe (MRS) broth (Oxoid, W. Heidelberg, Australia). To minimise carryover of free amino acids during inoculation, 5 mL of cells were washed and resuspended to the original volume with 0.32 mM sodium phosphate, pH 7.2. Cells were inoculated (1%) into RSM 12% (w/v) and incubated at their optimum temperatures (37°C for *S. thermophilus*, *L. acidophilus*, and *Bifidobacterium* spp. and 42°C for *L. delbrueckii* ssp. *bulgaricus*) for 6 h. A control consisted of uninoculated RSM. A 2.5 mL sample of each incu- bated RSM was mixed with 10 mL of 0.75 M trich- loroacetic acid (TCA) and 1 mL of water to 5 mL of sample to give a final concentration of 0.47 M (7.7%) TCA. The samples were filtered using a Whatman num- ber 4A filter paper after 10 min of incubation at room temperature (~22°C) and frozen at −80°C until assayed. The *o*-phthaldialdehyde (OPA) method described by Church, Swaisgood, Porter and Catignani (1983) was used to determine the concentration of free amino groups in the filtrate. Triplicate aliquots from each TCA filtrate were analysed using a Pharmacia LKB Novaspek II Spectrophotometer (Pharmacia, Biotech, Uppsala, Sweden).

2.3. Preparation of intracellular and cell wall extracts

The bacteria were grown three times successively in RSM to activate the organisms and finally in MRS broth for the lactobacilli, and ST broth for the *S. thermophilus* to avoid carryover of milk proteins. The growth was assessed by measuring the absorbance at 600 nm. The cells in the late logarithmic phase were collected from the growth medium by centrifugation at 12,000 *g* for 15 min at 4°C. The supernatant was designated the cell wall extracellular extract (EE). The pellet obtained was washed twice with 0.9% (w/v) NaCl solution. The pellet was resuspended in 0.05 M Tris-HCl buffer, pH 8.5 at 10% of volume of original growth medium and sonicated

30 s inervals for 5 min at 4°C. The supernatant obned after centrifugation (12,000 g for 15 min at 4°C) s designated as the intracellular extract (IE). The thod of Lowry, Rosebrough, Farr, and Randall (1951) is used (with bovine serum albumin as standard) to termine protein content in the extracellular and incellular extracts.

4. Enzyme assays

Aminopeptidase activity was measured using chromonic substrates (*p*-nitroanilide derivatives of L-anomers leucine, lysine, alanine, proline, arginine and methionine) according to the method of Fernandez-Espla, Martin-Hernandez, and Fox (1997). Aminopeptidase activity was assayed by incubation of 100 μL of sample with 00 μL of 50 mM Tris-HCl buffer, pH 7, and 50 μL of 0 mM of substrate at 37°C for 20 min. The reaction was topped by addition of 1 mL of 30% acetic acid. The release of *p*-nitroanilide was followed by measuring the bsorbance at 410 nm. The concentration of *p*-nitroanilide was calculated from the derived value of molar absorption coefficient ($\varepsilon = 9024\,mol^{-1}\,cm^{-1}$). One unit of enzyme activity was defined as the amount of enzyme equired to release 1 μmol of *p*-nitroanilide per min under he above conditions of the assay. The specific activity was expressed as units per milligram of protein.

Dipeptidase activity of the proteolytic strains of probiotic and yogurt bacteria was measured using Ala-Met, Leu-Tyr, Leu-Gly, Ala-His, and Pro-Ile as substrates according to the method of Wohlrab and Bockelmann (1992). The reaction mixture contained 10 μL of enzyme solution, 415 μL of 50 mM Tris-HCl, pH 7.5, 50 μL of 22 mM substrate, 25 μL of peroxidase (5 mg mL^{-1} in 0.8 M $(NH_4)_2SO_4$), 25 μL of L-amino acid oxidase (2 mg mL^{-1} in distilled water), and 25 μL of *o*-dianisidine (11.5 mM), respectively. The test tubes containing reaction mixture were incubated at 50°C for 20 min. The reactions were stopped by the addition of 50 μL of dithiothreitol (120 mM). Oxidation of *o*-dianisidine coupled to substrate hydrolysis resulted in an increase of brown colour which was measured at 436 nm. Enzyme activity was calculated by using a molar absorbance coefficient of 8100 mol^{-1}cm^{-1}. The specific activity was expressed as units per milligram of protein.

Endopeptidase and tripeptidase activities in the EE and IE extracts were detected by thin-layer chromatography as per the method of Tan and Konings (1990). The reaction mixture contained 2 mM substrate, 20 mM Tris-HCl, pH 7, and an appropriate amount of extract. The reaction mixture was incubated for 60 min at 37°C. The reaction was stopped by adding 10 μL of 30% acetic acid and the mixture was cooled to 4°C. Ten microlitres of the mixture was then spotted onto a precoated 0.25 cm-thick silica gel 60 plate (Merck, Darmstadt, Germany) and TLC was performed as described by Tan and Konings (1990). Peptides and amino acids became visible in UV light.

Unless otherwise indicated, all experiments and analyses were replicated 3 times and the results presented are averages of the nine replicates.

2.5. Statistical analysis

Data from the OPA analyses were subjected to one-way ANOVA, while the data from the enzymic analyses were subjected to two-way ANOVA (SigmaStat®, 1997).

3. Results and discussion

3.1. Proteolytic activity

The OPA-based spectrophotometric assay detects released α-amino groups, which result from the proteolysis of milk proteins, thus giving a direct measurement of proteolytic activity. Figs. 1 and 2 represent the proteolytic activities in RSM of 6 strains of *S. thermophilus*, 5 strains of *L. delbrueckii* ssp. *bulgaricus*, 12 strains of *L. acidophilus*, and 3 strains of *Bifidobacterium* spp., respectively. The proteolytic activity of these bacterial cultures is expressed as the amount of free amino groups measured as difference in absorbance values at 340 nm, after substraction of values for the uninocu label control RSM. The extent of proteolysis based upon OPA values was variable between *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* strains (Fig. 1), *L. acidophilus* and *Bifidobacterium* strains (Fig. 2) and appeared to be strain specific. These results have shown that the proteolytic activity of *S. thermophilus*, *L. delbrueckii* spp. *bulgaricus*, and *L.*

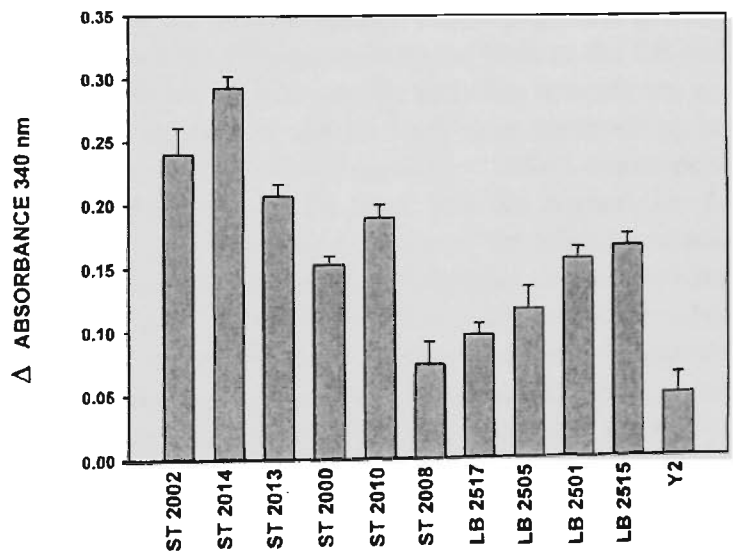

Fig. 1. Indication of proteolytic activity of *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* strains after incubation in RSM at 37 and 42°C, respectively for 6 h. Data represent differences in absorbance values after subtracting the value (1.68) for the control RSM.

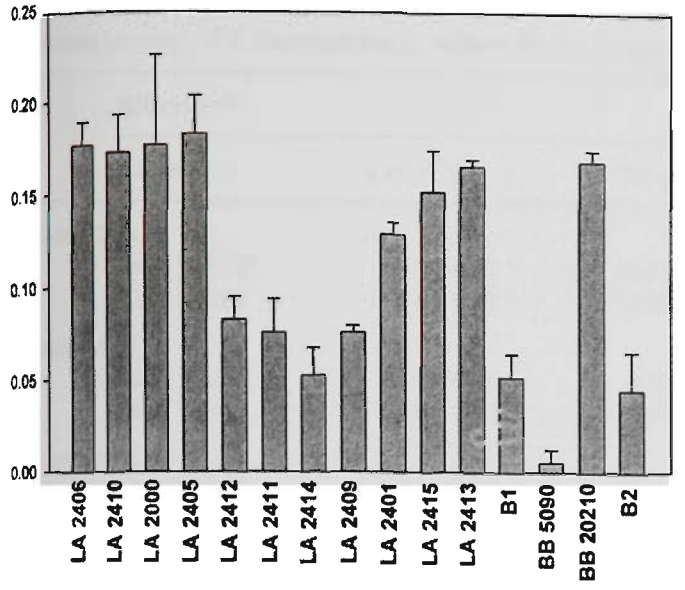

Fig. 2. Indication of proteolytic activity of *L. acidophilus* and *Bifidobacterium* spp. strains after incubation in RSM at 37°C for 6 h. Data represent differences in absorbance values after subtracting the value (1.68) for the control RSM.

acidophilus was much higher than that of *Bifidobacterium* spp., in agreement with Klaver et al. (1993), showing that *Bifidobacterium* strains are not highly proteolytic when compared to other lactic acid bacteria. Three *S. thermophilus* strains, one *L. delbrueckii* ssp. *bulgaricus* strain, two *L. acidophilus* strains and 10 *Bifidobacterium* strains did not show any proteolytic activity and hence those organisms are not represented in Figs. 1 and 2. *S. thermophilus* 2014 was highly proteolytic producing the highest amount of free amino groups (52.6 μM) among the nine strains studied, whereas the strains T-1, T-4, and Y-1 were not proteolytic. The differences in the mean values among the *S. thermophilus* strains are greater than would be expected so the proteolytic activities among the *S. thermophilus* strains were significantly different ($P < 0.001$).

L. delbrueckii ssp. *bulgaricus* strains were less proteolytic (Fig. 1) than *S. thermophilus* strains. *L. delbrueckii* ssp. *bulgaricus* strain 2515 showed the highest proteolytic activity releasing the highest amount of free amino groups (25 μM). The proteolytic activities among the *L. delbrueckii* ssp. *bulgaricus* strains were significantly different ($P < 0.001$). There was also a significant difference in proteolytic activity between the *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* ($P < 0.001$) groups based on the amount of free amino groups released.

Starter cultures containing *L. acidophilus*, *Bifidobacterium* spp., and *S. thermophilus* (ABT-cultures) are becoming popular in Australia. ABT-cultures lack *L. delbrueckii* ssp. *bulgaricus*, and as a result, the fermentation time for yogurt making is longer with these cultures (Dave & Shah, 1997). Dave and Shah (1998) observed a 3–4 log cycle drop in the counts of *Bifidobacterium* in an ABT starter culture; supplementation with acid casein hydrolysate, which contained peptides and amino acids improved the viability of *Bifidobacterium* spp.

Among the probiotic bacteria, *L. acidophilus*, in general, was more proteolytic than *Bifidobacterium* spp. (Fig. 2), and the differences in proteolytic activity between both groups were significantly different ($P < 0.001$). These results agree with the findings of Singh and Sharma (1983) and Koroleva et al. (1983) who reported high proteolytic activity of selected strains of *L. acidophilus*. Among bifidobacteria, *B. thermophilum* 20210 released appreciable amounts of free amino groups (30 μM). The rest of the strains did not release any amounts of free amino groups as compared to the control ($P < 0.001$), thus those strains could be classified as non-proteolytic. This may explain why bifidobacteria grow slowly in milk. It is presumed that free amino acids could be utilised during the early stage of incubation and that peptides could become available during the prolonged incubation of *Bifidobacterium* cultures (Cheng & Nagasawa, 1984). This may also explain why the growth of bifidobacteria requires supplementation of peptides and amino acids from external sources (Dave & Shah, 1998). The differences in the mean values among all bacterial groups were significant ($P < 0.001$) and greater than expected.

3.2. Enzyme assays

3.2.1. Aminopeptidase activity

Selected strains of *S. thermophilus*, *L. delbrueckii* ssp. *bulgaricus*, *L. acidophilus*, and *Bifidobacterium* spp. were further studied for their aminopeptidase, dipeptidase, tripeptidase, and endopeptidase activities, both at the extracellular (EE) and intracellular (IE) levels. *S. thermophilus* strains 2014, T-1, T-4, Y-1, *L. delbrueckii* ssp. *bulgaricus* strains 2515 and Y-2, *L. acidophilus* strains 2405 and B-1 and *B. thermophilum* 20210 and B-2 were selected for the enzyme assays. Aminopeptidase activity was detected for all bacterial strains, both at the EE and IE levels (Table 1). The specific activities towards the six substrates studied at the IE level were observed to be different to that at the EE level ($P < 0.001$). Aminopeptidase activity at the IE level was the highest for *L. delbrueckii* ssp. *bulgaricus* followed by *Bifidobacterium* spp., *S. thermophilus*, and *L. acidophilus*. *S. thermophilus* 2014 did not hydrolyse the substrate L-arginine-*p*Na ($P < 0.05$). At the EE level, *L. delbrueckii* ssp. *bulgaricus* 2515 showed higher aminopeptidase activity than *L. delbrueckii* ssp. *bulgaricus* Y-2 strain. However, the opposite was true when comparing activities at the IE level except for L-arginine-*p*Na. L-proline-*p*Na was greatly hydrolyzed by *L. delbrueckii* ssp. *bulgaricus* (Y-2) at the IE level which indicates the presence of iminopeptidase activity. *L. acidophilus* strains showed higher activities at the IE level than the EE level. It is interesting to note that *L. acidophilus* 2405 showed greater specificity towards

ble 1
inopeptidase activity[a] of *S. thermophilus*, *L. delbrueckii* ssp. *bulgaricus*, *L. acidophilus* and *Bifidobacterium* spp.

cterial itures	Substrates[b]					
	Leu-*p*Na	Lys-*p*Na	Pro-*p*Na	Ala-*p*Na	Meth-*p*Na	Arg-*p*Na
thermophilus 2014						
[c]	4.55 ± 6.36[d]	6.67 ± 0.30	10.61 ± 0.61	20.91 ± 2.10	16.06 ± 2.42	0.00 ± 0.00
[e]	27.04 ± 0.45	35.67 ± 0.46	17.89 ± 0.00	18.48 ± 1.47	29.33 ± 0.94	132.91 ± 7.82
thermophilus ABT-1						
E	2.43 ± 0.37	4.83 ± 0.07	6.43 ± 0.09	3.87 ± 0.14	4.30 ± 0.44	4.77 ± 0.07
E	6.07 ± 0.52	5.83 ± 1.74	7.37 ± 0.65	11.80 ± 1.04	7.63 ± 1.16	10.37 ± 0.61
thermophilus ABT-4						
E	2.90 ± 0.00	6.17 ± 0.18	5.63 ± 0.77	5.30 ± 0.19	4.83 ± 0.35	5.97 ± 0.37
E	18.23 ± 1.81	19.77 ± 1.58	12.23 ± 0.23	19.33 ± 0.38	12.87 ± 0.81	21.77 ± 0.79
thermophilus ABY-1						
E	3.27 ± 0.28	3.17 ± 0.07	5.30 ± 0.40	4.57 ± 0.10	4.50 ± 0.20	4.47 ± 0.03
E	6.57 ± 0.50	3.53 ± 0.59	5.93 ± 0.24	7.97 ± 0.24	5.27 ± 0.27	8.17 ± 0.23
bulgaricus 2515						
E	15.78 ± 1.00	9.39 ± 1.59	10.27 ± 0.27	19.46 ± 0.18	21.77 ± 0.27	148.14 ± 1.43
E	84.04 ± 2.75	129.76 ± 3.33	39.29 ± 1.80	46.90 ± 1.67	56.90 ± 3.92	185.13 ± 5.88
bulgaricus ABY-1						
E	5.07 ± 0.08	3.68 ± 0.28	4.17 ± 0.21	6.79 ± 0.50	3.76 ± 0.29	4.25 ± 0.09
E	129.67 ± 5.21	320.61 ± 16.31	293.73 ± 0.58	88.21 ± 5.23	75.06 ± 2.30	13.51 ± 1.58
acidophilus 2405						
E	7.94 ± 0.34	5.96 ± 0.28	5.98 ± 0.32	6.94 ± 0.02	8.21 ± 0.11	43.50 ± 1.72
E	21.40 ± 0.94	24.50 ± 1.37	15.81 ± 0.70	15.81 ± 0.48	21.24 ± 0.89	8.30 ± 0.26
L. acidophilus ABT-1						
EE	5.43 ± 0.10	7.00 ± 0.06	7.70 ± 0.66	6.60 ± 0.17	5.97 ± 0.07	6.70 ± 0.31
IE	21.43 ± 0.33	47.00 ± 3.51	13.33 ± 1.54	21.77 ± 0.72	14.20 ± 1.10	36.00 ± 1.85
Bifidobacterium spp. 20210						
EE	9.36 ± 0.06	7.67 ± 0.42	7.83 ± 0.29	8.13 ± 0.02	8.45 ± 0.18	328.27 ± 0.64
IE	94.33 ± 1.45	66.67 ± 1.45	87.33 ± 4.84	81.00 ± 0.58	98.33 ± 1.10	8.65 ± 0.09
Bifidobacterium spp. ABT-1						
EE	2.6 ± 0.10	4.97 ± 0.07	4.93 ± 0.09	4.37 ± 0.10	4.10 ± 0.10	4.57 ± 0.03
IE	10.67 ± 0.13	9.07 ± 0.35	10.00 ± 0.46	11.60 ± 0.46	10.67 ± 1.33	11.53 ± 1.41

[a]Aminopeptidase activity expressed as specific activity which is defined as units of enzyme activity per milligram of protein.
[b]Leu-*p*Na, Lys-*p*Na, Pro-*p*Na, Ala-*p*Na, Meth-*p*Na and Arg-*p*Na are *p*-nitroanilide derivatives of these amino acids.
[c]EE = cell wall extracellular extract.
[d]Values are means ± standard error.
[e]IE = intracellular extracts.

the substrate L-arginine-*p*Na at the EE level than at the IE level ($P < 0.05$). Also, *Bifidobacterium* spp. showed high levels of intracellular aminopeptidase activity but very little proteolytic activity. Desjardins, Roy, Toupin and Goulet (1990) found that the number of α-amino groups released during the exponential and stationary phases of growth steadily increased, but the rate of proteolysis did not follow the rate of biomass production. *B. thermophilum* 20210 demonstrated higher aminopeptidase activity both at the IE and EE levels ($P < 0.05$) than *Bifidobacterium* strain B-2 which was isolated from a commercial starter culture. L-methionine was the preferred substrate by *B. thermophilum* 20210 followed by L-leucine, L-proline, L-alanine, L-lysine and L-arginine.

3.2.2. Dipeptidase activity

Dipeptidase activities of *S. thermophilus*, *L. delbrueckii* ssp. *bulgaricus*, *L. acidophilus*, and *Bifidobacterium* spp. towards the five substrates selected are shown in Table 2. In general, *S. thermophilus* 2014 showed higher extracellular activity towards all five substrates studied, whereas the other strains showed variable specificity towards the substrates ($P < 0.05$). *L. delbrueckii* ssp. *bulgaricus* showed higher dipeptidase activity, towards all substrates except for Leu–Gly when compared to strain Y-2 ($P < 0.05$). Both lactobacilli, however, showed higher intracellular dipeptidase activity for four of the five substrates. Leu–Gly was greatly hydrolyzed by *S. thermophilus* T-4 at the EE level whereas the same applies for

ble 2
peptidase activity[a] of *S. thermophilus*, *L. delbrueckii* ssp. *bulgaricus*, *L. acidophilus* and *Bifidobacterium* spp.

terial cultures	Substrates				
	Ala-Met	Leu-Tyr	Pro-Ile	Leu-Gly	Ala-His
thermophilus 2014					
b	1100 ± 6.41[c]	992.73 ± 6.88	948.48 ± 13.21	7.86 ± 0.73	576.00 ± 11.65
d	94.22 ± 1.63	120.44 ± 9.77	14.81 ± 0.53	3.94 ± 0.29	11.79 ± 0.78
thermophilus					
BT-1					
E	28.40 ± 1.90	26.93 ± 1.68	61.93 ± 2.99	17.02 ± 1.02	13.83 ± 2.24
E	60.00 ± 3.13	56.90 ± 3.25	10.97 ± 0.99	13.83 ± 2.24	33.95 ± 0.63
thermophilus					
BT-4					
E	30.73 ± 1.85	35.90 ± 0.46	86.53 ± 0.83	18.84 ± 0.55	22.89 ± 0.97
E	91.77 ± 5.03	97.10 ± 4.83	13.10 ± 0.20	22.89 ± 0.97	37.78 ± 2.74
thermophilus					
BY-1					
E	20.57 ± 0.23	30.23 ± 1.98	47.57 ± 4.42	15.97 ± 0.76	11.25 ± 0.44
E	24.20 ± 1.84	59.90 ± 0.55	5.37 ± 0.52	11.25 ± 0.44	12.34 ± 0.16
bulgaricus 2515					
EE	173.73 ± 5.59	154.27 ± 8.75	182.40 ± 5.31	1.40 ± 0.09	97.72 ± 2.23
E	1270.48 ± 8.66	884.76 ± 11.38	135.71 ± 1.65	2.43 ± 0.09	438.29 ± 0.41
bulgaricus ABY-1					
EE	8.96 ± 0.42	8.57 ± 1.24	8.99 ± 0.96	6.49 ± 0.55	58.46 ± 0.99
E	9.30 ± 0.43	8.88 ± 0.81	38.06 ± 0.88	38.06 ± 0.88	63.97 ± 1.70
acidophilus 2405					
EE	46.37 ± 1.82	43.63 ± 1.50	49.80 ± 0.98	0.46 ± 0.03	30.05 ± 0.95
E	126.67 ± 8.24	105.76 ± 2.81	22.88 ± 0.66	0.82 ± 0.03	106.11 ± 1.19
acidophilus ABT-1					
EE	18.57 ± 1.40	17.33 ± 1.43	39.30 ± 0.46	7.60 ± 0.33	23.58 ± 1.00
E	36.83 ± 7.53	84.73 ± 0.32	15.27 ± 1.57	23.58 ± 1.00	26.19 ± 1.09
Bifidobacterium spp 20210					
EE	41.10 ± 1.11	40.59 ± 0.91	37.95 ± 3.25	0.48 ± 0.04	20.60 ± 1.96
E	1304.00 ± 8.33	1074.00 ± 9.45	90.00 ± 4.16	2.67 ± 0.14	141.20 ± 6.80
Bifidobacterium spp ABT-1					
EE	12.57 ± 0.09	11.67 ± 0.52	25.63 ± 0.23	5.08 ± 0.27	13.04 ± 0.31
E	28.40 ± 2.20	37.73 ± 3.93	9.87 ± 0.53	13.04 ± 0.31	12.79 ± 0.30

[a]Dipeptidase activity expressed as specific activity which is defined as units of enzyme activity per milligram of protein.
[b]EE = extracellular cell wall extract.
[c]Values are means ± standard error.
[d]IE = intracellular extract.

L. delbrueckii ssp. *bulgaricus* Y-1 at the IE level ($P < 0.001$). *B. thermophilum* 20210 showed the highest activity towards the same substrates at the IE level. *L. acidophilus* 2405 showed similar activity when compared to the strain isolated from the commercial starter culture.

3.2.3. Endo- and tripeptidase activity

All bacterial cultures tested were able to hydrolyse the large biologically active peptides, bradykinin and Ala-Ala-Ala-Ala-Ala at both the extracellular and intracellular levels. The same can be deduced for the tripeptide substrate Gly-Ala-Tyr (Table 3). However, with the substrates ending with a C-terminal of phenylalanine, Gly-Leu-Phe, and Gly-Gly-Phe, the hydrolysis only occurred at the IE level. This suggests the presence of a tripeptidase at the IE level.

4. Conclusions

The yogurt bacteria (*S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus*) appeared to be highly proteolytic as compared to the probiotic bacteria (*L. acidophilus* and *Bifidobacterium* spp.). The yogurt bacteria released higher

ble 3
peptidase and endopeptidase activities[a] of *S. thermophilus*, *L. delbrueckii* ssp. *bulgaricus*, *L. acidophilus*, and *Bifidobacterium* spp.

bstrates	Hydrolysis[b]	
	CWE[c]	IE[d]
ipeptides		
y-Ala-Tyr	+	+
y-Leu-Phe	−	+
ly-Gly-Phe	−	+
idopeptides		
radykinin	+	+
la-Ala-Ala-Ala	+	+

[a]Hydrolysis of peptides was analysed by TLC.
[b]+ = hydrolysis; − = no hydrolysis.
[c]CWE = cell wall extract.
[d]IE = intracellular extract.

mounts of free amino acids and demonstrated greater minopeptidase and dipeptidyl activity than the robiotic bacteria. As a result, yogurt bacteria grow aster in milk, whereas the probiotic bacteria grow slowly lue to lack of proteolytic activity and require an xogenous supply of peptides and amino acids for optinum growth, in particular for starter cultures that do not ontain *L. delbrueckii* spp. *bulgaricus*.

Acknowledgements

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Influence of addition of proteolytic strains of *Lactobacillus delbrueckii* subsp. *bulgaricus* to commercial ABT starter cultures on texture of yoghurt, exopolysaccharide production and survival of bacteria

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Abstract

The effects of addition of proteolytic strains of *Lactobacillus delbrueckii* subsp. *bulgaricus* to commercial ABT starter cultures on the texture of yoghurt, exopolysaccharide production and survival of starter bacteria were studied. The firmness of set yoghurts was measured with a cone-penetrometer and the apparent viscosity with a Brookfield viscometer. Exopolysaccharides (EPS) was extracted by precipitation with ethanol. Supplementation of a proteolytic strain of *L. delbrueckii* subsp. *bulgaricus* 2501 or 2515 to ABT-1 or ABT-4 starter culture reduced the fermentation time in making yoghurt. Yoghurts made with ABT-1 and ABT-4 with added *L. delbrueckii* subsp. *bulgaricus* 2501 fermented in the shortest time of 5 h and 33 min and 5 h and 8 min, respectively. Quantities of EPS extracted from the different yoghurt batches did not show any significant differences ($P<0.222$). In general, the firmness improved with the addition of the proteolytic strains of *L. delbrueckii* subsp. *bulgaricus*. Addition of *L. delbrueckii* subsp. *bulgaricus* to ABT-1 and ABT-4 did not produce significant ($P<0.001$) improvement in firmness of yoghurt compared with the mix starter batches. Significant differences ($P<0.001$) were observed in the viscosity of yoghurts supplemented with different combinations of starter cultures. However, the amounts of EPS isolated from yoghurt cultures did not correlate with the viscosities of the yoghurts. Viability of probiotic bacteria improved in the yoghurt product made with mix starter culture with the added *L. delbrueckii* subsp. *bulgaricus* proteolytic strains.

Keywords: Exopolysaccharide; Probiotic bacteria; Yoghurt bacteria

1. Introduction

Many functional characteristics of lactic acid bacteria (LAB) are responsible for their historical and modern use in food production. One such characteristic that has recently generated much interest is polysaccharide production. LAB are capable of producing several different polysaccharides, which can be found in the cytoplasm as carbon and energy sources, as components of the bacterial cell wall, or as external appendages of the cell. Exopolysaccharides (EPS) are polysaccharides external to the cell, either attached to cells or excreted as free polysaccharides (Cerning, 1990; Cerning, Bouillanne, Landon, & Desmazeaud, 1992; Nakajima et al., 1990). Nomenclature for describing EPS isolated from LAB is ambiguous. They have been described as mucoid, slime producing, and ropy, and their compositions and structures are very heterogeneous (Knoshaug, Ahlgrent, & Trempy, 2000).

Ropy EPS-producing LAB strains have been used in Scandinavian countries to produce a thick fermented milk called viili, langfil, or filmjolk (Wacher-Rodarte et al., 1993). Currently, EPS-producing LAB strains are used in yoghurt, sour cream and whipped toppings to improve their rheological properties, to prevent syneresis, and to replace stabilizers.

The use of *Lactobacillus acidophilus* and bifidobacteria (AB) bacteria in yoghurt is increasing due to potential health benefits associated with the consumption of these bacteria. Viability of AB bacteria is of paramount importance in marketing of probiotic products. However, in a previous study (Dave & Shah, 1997b), viability

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of bifidobacteria declined dramatically during manufacture of yoghurt with *Lactobacillus acidophilus*, bifidobacteria and *Streptococcus thermophilus* (ABT) starter culture that produced EPS. Use of exopolysaccharide-producing yoghurt starter cultures is gaining popularity among yoghurt manufacturers (Wacher-Rodarte et al., 1993; Hess, Roberts, & Ziegler, 1997). Gassem, Schmidt, and Frank (1995) and Griffin, Morris, and Gasson (1996) reported that polysaccharide-producing yoghurt bacteria were important determinants of yoghurt viscosity and texture. These starter cultures improve the viscosity of yoghurt leading to resistance to mechanical damage (Tamime & Deeth, 1980). The polysaccharide-producing ABT yoghurt starter cultures have established their market in Australia and Europe.

Hassan, Frank, Schmidt, and Shalabi (1996) studied the textural properties of yoghurt made with encapsulated ropy and non-ropy yoghurt cultures and concluded that the firmness and viscosity were dependent on the pH of yoghurt and the type of starter culture used to manufacture the product. Polysaccharide-producing bacteria are reported to produce yoghurt gels of low fracturability and the apparent viscosity was dependent on the capsule size and bacterial counts (Kailasapathy, Supriadi, & Hourigan, 1996). Exopolysaccharide cultures of LAB have been recognized for many years as the cause of the viscosity of ropy milk (Escalante, Wacher-Rodarte, Garcia-Garibay, & Farres, 1998). Use of slime-producing strains of *S. thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*, especially in stirred yoghurts, results in a thicker body and higher viscosity, enhanced smoothness, and in prevention of syneresis (Wacher-Rodarte et al., 1993).

In ABT, *S. thermophilus* is the sole fermenting organism. No symbiotic relationship exists during fermentation as *L. delbrueckii* subsp. *bulgaricus* is absent from the starter culture. The three bacteria used in ABT starter culture are reported to be less proteolytic than *L. delbrueckii* subsp. *bulgaricus* (Shihata & Shah, 2000). As a result, the fermentation is slow with ABT starter cultures and the viability of AB organisms is reported to be low as the organisms require additional nutrients such as amino acids and peptides (Dave & Shah, 1998). Therefore, the addition of proteolytic *L. delbrueckii* subsp. *bulgaricus* to these starter cultures might improve the fermentation process and the viability of AB organisms as the former may release amino acids and peptides through breakdown of protein in milk for the latter.

Therefore, in the present study the effects of addition of proteolytic strains of *L. delbrueckii* subsp. *bulgaricus* to commercial ABT starter cultures on the texture of yoghurt, exopolysaccharide production and viability of probiotic organisms were studied.

2. Materials and methods

2.1. Bacterial cultures

S. thermophilus-1 (ST-1), *S. thermophilus*-4 (ST-4), *L. acidophilus*-1 (LA-1), *L. acidophilus*-4 (LA-4), bifidobacteria-1 (BB-1) and bifidobacteria-4 (BB-4) were isolated from ABT-1 and ABT-4 commercial starter cultures (Chr. Hansen Pty. Ltd., Bayswater, Australia). The organisms were propagated separately. *L. delbrueckii* subsp. *bulgaricus* strains 2501 and 2515 were obtained from the Victoria University culture collection (Melbourne, Vic., Australia) and were selected based on their high proteolytic activity as reported earlier (Shihata & Shah, 2000). Each culture was maintained in 12% sterile reconstituted skim milk (RSM) supplemented with 2% glucose and 1% yeast extract. All cultures were stored at −80°C and working cultures were propagated successively three times before use and stored at 4°C.

2.2. Yoghurt preparation

Homogenised and pasteurised milk (2 L) was heated to 85°C for 30 min, cooled to 43°C and different combinations of bacterial cultures (1% of yoghurt mix) were added as shown in Table 1. The mix was distributed in 500 mL (for firmness and viscosity studies) and 100 mL plastic cups (for other analyses). Incubation was carried out at 42°C and fermentation was terminated at pH 4.5. After fermentation, yoghurt samples were removed and stored at 4°C for 4 weeks.

Table 1
Combination of starter cultures used in yoghurt preparations

Starter culture used for yoghurt production	Bacterial species
ABT[a]-1	ST[b]-1, LA[c]-1 and BB[d]-1
ABT-1 + LB[e] 2501	ST-1, LA-1, BB-1 and LB 2501
ABT-1 + LB 2515	ST-1, LA-1, BB-1 and LB 2515
ABT-4	ST-4, LA-4 and BB-4
ABT-4 + LB 2501	ST-4, LA-4, BB-4 and LB 2501
ABT-4 + LB 2515	ST-4, LA-4, BB-4 and LB 2515
Mix starter culture	ST 2002, LA 2415 and BB 20210
Mix starter culture + LB 2501	ST 2002, LA 2415, BB 20210 and LB 2501
Mix starter culture + LB 2515	ST 2002, LA 2415, BB 20210 and LB 2515

[a] ABT = *L. acidophilus*, *Bifidobacterium* sp. and *S. thermophilus*.
[b] ST = *S. thermophilus*.
[c] LA = *L. acidophilus*.
[d] BB = *Bifidobacterium* sp.
[e] LB = *L. delbrueckii* subsp. *bulgaricus*.

2.3. Time interval specifications

The '0 day' (day 0) analyses were carried out after overnight cold storage of yoghurt samples, and 'week 1', 'week 2', 'week 3' and 'week 4' analyses were carried out after 7, 14, 21 and 28 days of storage, respectively.

2.4. Sample preparation

Yoghurt prepared in 500 mL cups was used for measuring the firmness of the product at day 0. After measuring the firmness, the yoghurt sample was uniformly mixed and ≈450 mL sample taken in 500 mL glass beakers for the measurement of viscosity. All the measurements were carried out in triplicate.

2.5. Texture analyses

The firmness of the set yoghurt at day 0 was measured with a cone-penetrometer (Stanhope-Seta Ltd., Surrey, England). The first and second angles of the cone were at 30° and 90°, respectively, and the total weight of the cone with its holder was 102.52 g. The depth of cone penetration was measured in millimetres after 5 s of cone penetration. All analyses in triplicate were carried out at 4°C.

The apparent viscosity of yoghurt samples at 0 day was measured with a Brookfield viscometer (Model DV II, Brookfield Engineering Laboratory, Stoughton, USA) using a Helipath D spindle at 1.5 rpm. Yoghurt samples were maintained at 4°C. Yoghurt, being a non-Newtonian fluid, gave no stable reading of viscosity, therefore, the data presented are the averages of the highest and the lowest viscosity readings in centipoise obtained for each sample. The average range of fluctuations in viscosity readings was about 5–10%. For the viscosity measurements, a homogenously mixed yoghurt sample was filled in a 500 mL glass beaker and the spindle allowed to spin in the sample.

2.6. Extraction and quantification of exopolysaccharide

Exopolysaccharide from yoghurts was extracted according to method of Uemura, Itoh, Kaneko, and Noda (1998). The yoghurt sample (100 g) was diluted twice with distilled water (200 mL). Casein was precipitated by adjusting to pH 4.6 with 2 M NaOH solution and removing it together with the microorganisms by centrifugation (10,000*g*, 4°C for 20 min). After neutralization of the whey fraction with the additional NaOH solution, the sample was heated for 30 min in a boiling water bath, and the insolubilized protein was removed by centrifugation (10,000*g*, 4°C, 20 min). After the addition of an equal volume of cold ethanol (99.5%), the solution was stirred at 4°C overnight, and the resulting precipitate was recovered by centrifugation and then dissolved in distilled water. The precipitation procedure with ethanol was repeated twice. The separated crude polysaccharide was resuspended in 0.05 M Tris-HCl buffer (pH 8.0) containing 1 mM $MgCl_2$ and then treated with 2 μg mL^{-1} of DNase and RNase each at 37°C, for 6 h. The contaminating protein in the sample was digested with 0.2 mg mL^{-1} of proteinase K overnight at 37°C. After stopping the reaction by using heat at 90°C for 10 min, the polysaccharide fraction was precipitated with ethanol and dialysed with distilled water, and then lyophilised (exocellular polysaccharide; EPS) and weighed. Total EPS was assayed and the quantification of sugar was carried out using the method of Dubois, Gilles, Hamilton, Rebers, and Smith (1956). The amount of EPS was expressed as microgram of glucose equivalents per gram of yoghurt using glucose as a standard.

2.7. Microbiological analyses

One gram of yoghurt sample was diluted with 9 mL of 1.5% peptone and diluent water (Oxoid, W. Heidelberg, Australia) and mixed uniformly. Subsequent serial dilutions were prepared and viable numbers enumerated using pour plate technique. The counts of *S. thermophilus* were enumerated on ST agar by incubating the plates aerobically at 37°C for 24 h. MRS agar (Oxoid, Australia) adjusted to pH 5.2 and anaerobic incubation at 43°C for 72 h were used for the differential enumeration of *L. delbrueckii* subsp. *bulgaricus*. *L. acidophilus* and *Bifidobacterium* sp. were differentially enumerated using MRS-sorbitol and MRS-NNLP (nalidixic acid, neomycin sulfate, lithium chloride and paramomycin sulfate) agar, respectively, and incubated anaerobically at 37°C for 72 h (Dave & Shah, 1996).

Unless otherwise indicated, all experiments and analyses were replicated three times and results presented are averages of the nine replicates.

2.8. Statistical analysis

The data was subjected to one-way ANOVA analysis (SigmaStat®, 1997).

3. Results and discussion

The ABT starter culture consists of *L. acidophilus*, *Bifidobacterium* sp. and *S. thermophilus*. Previous studies have shown low viability of *Bifidobacterium* sp. and longer fermentation times for yoghurt production with ABT starter culture (Dave & Shah, 1997a, b). Therefore, this study investigated the effects of addition of proteolytic strains of *L. delbrueckii* subsp. *bulgaricus* (Shihata & Shah, 2000) on fermentation time, EPS production, textural properties of yoghurts and viability

Table 2
Fermentation times for preparation of yoghurt to reach pH 4.5, and EPS contents of various types of yoghurts

Yoghurt sample	Fermentation times	EPS (g $100\,g^{-1}$)
ABT[a]-1	10 h 7 min	3.01 ± 0.36
ABT-1 + LB 2501	5 h 33 min	3.41 ± 0.34
ABT-1 + LB 2515	6 h 25 min	3.61 ± 0.45
ABT-4	6 h 0 min	2.76 ± 0.36
ABT-4 + LB[b] 2501	5 h 8 min	3.27 ± 0.31
ABT-4 + LB 2515	6 h 25 min	3.56 ± 0.12
Mix starter culture[c]	12 h 0 min	3.01 ± 0.08
Mix + LB 2501	7 h 0 min	2.88 ± 0.19
Mix + LB 2515	6 h 47 min	3.64 ± 0.20

[a] ABT = *L. acidophilus*, *Bifidobacterium* sp. and *S. thermophilus*.
[b] LB = *L. delbrueckii* subsp. *bulgaricus*.
[c] Mix starter culture = *S. thermophilus* 2002, *L. acidophilus* 2415 and *Bifidobacterium* 20210.

of *S. thermophilus*, *L. delbrueckii* subsp. *bulgaricus*, *L. acidophilus* and *Bifidobacterium* sp.

The fermentation time and EPS contents of different yoghurts made with various starter culture combinations are presented in Table 2. In general, addition of *L. delbrueckii* subsp. *bulgaricus* 2501 to ABT starter cultures performed better than *L. delbrueckii* subsp. *bulgaricus* 2515 in reducing fermentation time by 50% when compared to ABT-1 alone. The fermentation times with ABT-1 and ABT-4 starter cultures were 10 h and 7 min and 6 h, respectively. Yoghurts made with ABT-1 and ABT-4 starter cultures with added *L. delbrueckii* subsp. *bulgaricus* 2501 fermented in the shortest time of 5 h and 33 min and 5 h and 8 min, respectively. The yoghurts made with the starter cultures ABT-1 and ABT-4 with added *L. delbrueckii* subsp. *bulgaricus* 2515, respectively, were the third quickest to ferment in 6 h and 25 min. The mix starter culture (Table 2) with the added *L. delbrueckii* subsp. *bulgaricus* strains (2515 and 2501) showed fermentation times of 6 h, 47 min and 7 h, respectively. The slowest fermentation of 12 h was with the mix starter bacteria, which consisted of *S. thermophilus* 2002, *L. acidophilus* 2415, and bifidobacteria 20210. Thus, it appears that addition of proteolytic strain of *L. delbrueckii* subsp. *bulgaricus* helped reduce fermentation time, possibly due to liberation of peptides and amino acids required by less proteolytic bacteria.

The strains of *S. thermophilus* in ABT-1 starter culture and ABT-4 starter culture are different (Dave & Shah, 1997b). *S. thermophilus* in ABT-4 starter culture is used for fast set and ABT-1 is for slow fermentation and *S. thermophilus* strain in ABT-4 starter culture may be more proteolytic than that in ABT-1 starter culture (Chr. Hansen, personal communication). This could be the reason why there was no major change in the fermentation time when proteolytic strains of *L. delbrueckii* subsp. *bulgaricus* were added to ABT-4 starter culture. The fermentation time decreased by 4 h and 34 min with ABT-1 starter culture supplemented with *L. delbrueckii* subsp. *bulgaricus* 2501 as compared with a drop in the fermentation time of 52 min only with ABT-4 starter culture with the same strain of *L. delbrueckii* subsp. *bulgaricus*.

S. thermophilus strains are used in combination with *L. delbrueckii* subsp. *bulgaricus* strains as commercial yoghurt starters. However, in ABT starter culture, *S. thermophilus* is the sole fermenting organism. In yoghurt making, *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* grow symbiotically producing essential amino acids for each other and the fermentation time is ≈4 h. However, no symbiotic relationship exists during yoghurt making, as *L. delbrueckii* spp. *bulgaricus* is absent from the starter culture. As a result, the fermentation is slow with ABT starter cultures. Supplementation with amino acid and peptides is reported to improve the fermentation process with ABT starter culture and, also, viability of AB organisms (Dave & Shah, 1997b, 1998).

3.1. EPS quantification

EPS produced in yoghurt by the different yoghurt starter cultures was extracted with NaOH, precipitated with ethanol, followed by treatments with nuclease and proteinase. The quantity in gram of EPS per 100 g of yoghurt is shown in Table 2. The various mean values of EPS produced among the yoghurts made were not statistically different ($P<0.222$). In general, the quantities of EPS produced by these strains were higher when compared to literature values. Amounts of EPS from *S. thermophilus* can range from as little as $30\,mg\,L^{-1}$ (Cerning, Bouillanne, Desmazeaud, & Landon, 1988) to $890\,mg\,L^{-1}$ (Escalante et al., 1998), and $60–150\,mg\,L^{-1}$ for *L. delbrueckii* subsp. *bulgaricus* strains (Cerning, 1995; Cerning, Bouillane, Desmazeaud, and Landon (1986)). This may be due to the associative growth of the starter cultures used in this study, where all three bacterial cultures, *S. thermophilus*, *L. delbrueckii* subsp. *bulgaricus* and *Bifidobacterium* sp. were exopolysaccharide producers. Bouzar, Cerning, and Desmazeaud (1997) also reported faster EPS production when mixed cultures were used. The yield of EPS produced by LAB also depends on the composition of the medium (carbon and nitrogen sources) and the conditions in which the organisms are grown (De Vuyst & Degeest, 1999).

3.2. Texture analyses

3.2.1. Firmness

The firmness of yoghurt (measured as depth of penetration in millimetre) supplemented with *L. delbrueckii* subsp. *bulgaricus* strains 2501 and 2515 is shown in Fig. 1. In general, the firmness increased with

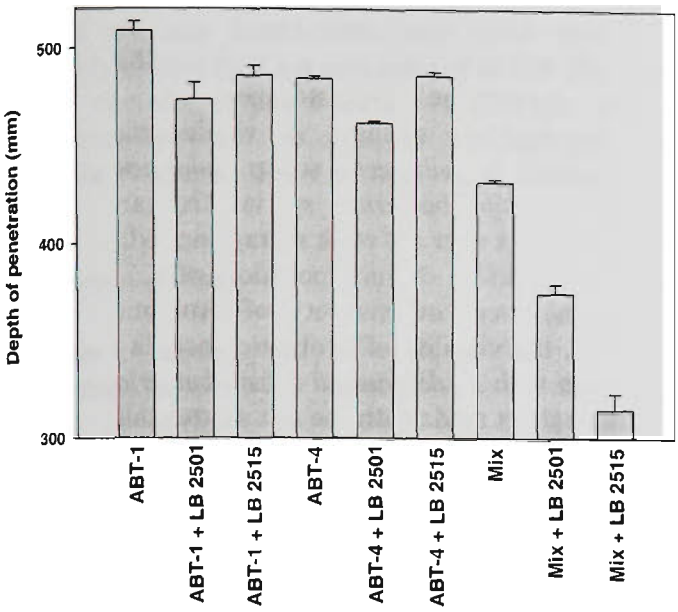

Fig. 1. Firmness of yoghurt measured as penetration depth (mm) affected by supplementation of *L. delbrueckii* subsp. *bulgaricus* strains (ABT = *L. acidophilus*, *Bifidobacterium* sp. and *S. thermophilus*; ST = *S. thermophilus*; LA = *L. acidophilus*; BB = *Bifidobacterium* sp.; LB = *L. delbrueckii* subsp. *bulgaricus*).

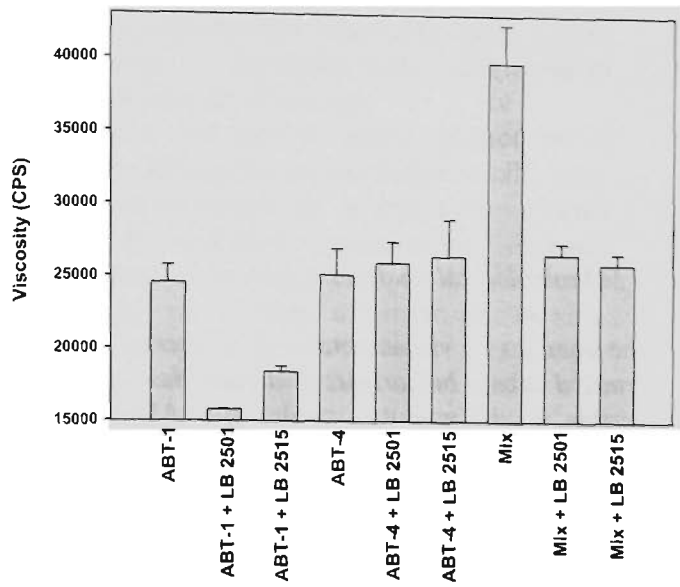

Fig. 2. Viscosity of yoghurt samples after supplementation of *L. delbrueckii* subsp. *bulgaricus* strains (ABT = *L. acidophilus*, *Bifidobacterium* sp. and *S. thermophilus*; ST = *S. thermophilus*; LA = *L. acidophilus*; BB = *Bifidobacterium* sp.; LB = *L. delbrueckii* subsp. *bulgaricus*).

incorporation of *L. delbrueckii* subsp. *bulgaricus* 2501 to ABT-1 and ABT-4 starter cultures. Yoghurts made with incorporation of *L. delbrueckii* subsp. *bulgaricus* 2501 to ABT-1 or ABT-4 starter cultures were firmer than those with *L. delbrueckii* subsp. *bulgaricus* 2515. The firmness of yoghurts made with ABT-1 starter culture was the lowest ($P<0.001$). Addition of *L. delbrueckii* subsp. *bulgaricus* 2515 to mix culture resulted in a significant ($P<0.001$) firm yoghurt. The depth of cone penetration was 314.7 and 374.7 mm, for mix culture with added *L. delbrueckii* subsp. *bulgaricus* 2515 and mix culture with added *L. delbrueckii* subsp. *bulgaricus* 2501, respectively, as compared to 431.7 mm for the yoghurt made with the mix starter culture only. The improvement in firmness with addition of *L. delbrueckii* subsp. *bulgaricus* to the mix culture was better ($P<0.001$) than that with ABT starter cultures. Improvement in firmness, in general, is due to the attachment of mucogenic strains to the protein matrix via the EPS (Marshall & Rawson, 1999).

3.2.2. *Apparent viscosity*

Apparent viscosity of yoghurt samples during refrigerated storage at day "0" is shown in Fig. 2. Significant differences ($P<0.001$) were observed in the viscosity of yoghurts supplemented with different combinations of starter cultures. The most viscous yoghurt produced was with mix starter cultures. The addition of *L. delbrueckii* subsp. *bulgaricus* 2501 or 2515 to ABT-1 or to the mix starter culture decreased the viscosity of yoghurt. *L. delbrueckii* subsp. *bulgaricus* 2501 and 2515 are reported to be proteolytic strains (Shihata & Shah, 2000). Proteolytic strains of *L. delbrueckii* subsp. *bulgaricus* are capable of hydrolysing proteins and this may have led to the reduction in viscosity of yoghurts. There appeared to be a relationship between the fermentation time and viscosity of yoghurt; as fermentation time increased, the viscosity increased. Upon addition of proteolytic strains of *L. delbrueckii* subsp. *bulgaricus*, the viscosity decreased. This suggests that the fermentation time could be reduced (Table 2) by incorporating proteolytic strains of *L. delbrueckii* subsp. *bulgaricus*; however, this may have adverse effects on viscosity of the product.

The amounts of EPS isolated from yoghurt made with various cultures (Table 2) did not correlate with the viscosities of the products. This agrees with previous studies, which stated that the apparent viscosity, which increases with increasing ropiness, was not simply related to the EPS concentration (Faber, Zoon, Kamerling, & Vliegenthart, 1998). Furthermore, Wacher-Rodarte et al. (1993) could not detect any statistically significant correlation between EPS-production and viscosity. However, Cerning et al. (1986, 1988) reported a relationship between viscosity and the amount of EPS produced, although this relationship was not always clear.

The quantities of polymer formed by ropy strains of bacterial cultures vary considerably even under identical experimental conditions (Cerning et al., 1990). It is difficult to establish a good correlation between the quantity of polysaccharide produced and the corresponding viscosity. This difficulty may be due to changes in the three-dimensional configuration of

polymers and to their interactions with some milk proteins, mainly caseins that are precipitated at low pH. In addition, viscosity measurements are difficult to interpret for non-Newtonian solutions such as milk and fermented milk products (Zourari, Accolas, & Desmazeaud, 1992).

3.3. Microbiological analyses

The changes in viable counts of *S. thermophilus*, *L. delbrueckii* subsp. *bulgaricus*, *L. acidophilus* and *Bifidobacterium* sp. in yoghurts made with ABT-1 starter cultures supplemented with *L. delbrueckii* subsp. *bulgaricus* 2501 or 2515 over a 4 week storage period are shown in Table 3. There was almost a 2 log increase in the population of *S. thermophilus* during fermentation. *S. thermophilus* in all yoghurt cultures was stable over the 4 week storage period. *L. delbrueckii* subsp. *bulgaricus*, *L. acidophilus* and *Bifidobacterium* sp. showed a decline in their numbers by week 4. The latter three organisms were able to show a relatively high viable count after 24 h but their numbers declined during storage. *L. acidophilus* in ABT-1 showed the lowest viable count. The addition of *L. delbrueckii* subsp. *bulgaricus* strains (2501 and 2515) improved the viability of probiotic bacteria (*L. acidophilus* and bifidobacteria) when compared with the control yoghurt culture (ABT-1).

Table 4 shows changes in viable counts of *S. thermophilus*, *L. delbrueckii* subsp. *bulgaricus*, *L. acidophilus* and *Bifidobacterium* sp. in ABT-4 starter culture based yoghurts over a 4 week storage period. There was no improvement in the counts of probiotic bacteria (*L. acidophilus* or bifidobacteria); however, the counts of bifidobacteria were ≈3 log cycles higher than those of *L. acidophilus* at the end of storage.

Table 5 shows changes in viable counts of *S. thermophilus*, *L. delbrueckii* subsp. *bulgaricus*, *L. acidophilus* and *Bifidobacterium* sp. in mix starter culture based yoghurts over a 4 week storage period. There was no positive effect of incorporation of *L. delbrueckii* subsp. *bulgaricus* on viability of probiotic bacteria. However, the viability of probiotic bacteria on supplementation with *L. delbrueckii* subsp. *bulgaricus* was the best in yoghurt made with the mix starter culture (Table 5) followed by that with ABT-4 (Table 4) and ABT-1 (Table 3) starter cultures. Dave and Shah (1997a) have shown that the probiotic organisms survived better in ABY (*L. acidophilus*, bifidobacteria and yoghurt cultures) starter cultures, which contain *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* than in ABT (*L. acidophilus*, bifidobacteria and *S. thermophilus*) starter cultures. In the present investigation, ABT-4 (Table 4) showed better viability of probiotics than ABT-1 starter culture (Table 3). This is in agreement with the previous study of Dave and Shah (1997b). ABY starter cultures contain yoghurt bacteria, in particular *L. delbrueckii* subsp. *bulgaricus*, which is more proteolytic than *S. thermophilus*. Symbiotic growth also occurs due to the presence of both bacterial species where they can produce amino acids and peptides, which are required for growth and survival of probiotic bacteria. Supplementation with amino acids and peptides through acid casein hydrolysates has also shown to improve the survival of probiotic organisms (Dave & Shah, 1998).

Table 3
Changes in viable counts of *S. thermophilus*, *L. delbrueckii* subsp. *bulgaricus*, *L. acidophilus* and *Bifidobacterium* sp. in ABT-1 starter cultures during manufacture and storage of yoghurt (ABT = *L. acidophilus*, *Bifidobacterium* sp. and *S. thermophilus*; ST = *S. thermophilus*; LA = *L. acidophilus*; BB = *Bifidobacterium* sp.; LB = *L. delbrueckii* subsp. *bulgaricus*)

	0 h	24 h	Week 1	Week 2	Week 3	Week 4
ST-1	6.93±0.02	8.95±0.06	9.08±0.02	8.93±0.03	9.25±0.02	9.13±0.02
LA-1	4.20±0.01	6.78±0.00	6.00±0.02	3.91±0.00	3.69±0.04	2.59±0.03
BB-1	4.10±0.05	5.89±0.01	5.81±0.00	4.64±0.04	4.58±0.01	2.44±0.04
ST-1[a] (ABT-1 + LB 2501)	6.92±0.02	9.12±0.01	9.31±0.01	9.11±0.01	9.18±0.01	9.25±0.03
LB 2501	4.81±0.00	8.01±0.02	6.88±0.00	6.34±0.02	6.15±0.04	4.63±0.03
LA-1[b] (ABT-1 + LB 2501)	4.27±0.01	6.19±0.01	4.29±0.02	3.99±0.01	3.91±0.01	3.81±0.03
BB-1[c] (ABT-1 + LB 2501)	4.10±0.01	5.00±0.01	6.08±0.04	3.68±0.08	3.54±0.08	3.48±0.02
ST-1[d] (ABT-1 + LB 2515)	6.93±0.01	9.20±0.00	9.27±0.04	9.10±0.00	9.22±0.04	9.21±0.04
LB 2515	4.57±0.04	7.75±0.03	7.71±0.01	7.10±0.01	6.13±0.00	5.14±0.02
LA-1[e] (ABT-1 + LB 2515)	4.20±0.08	5.94±0.02	4.05±0.01	3.94±0.03	3.85±0.01	3.72±0.01
BB-1[f] (ABT-1 + LB 2515)	4.08±0.01	5.17±0.03	3.89±0.01	3.66±0.03	3.87±0.05	2.73±0.02

[a] ST counts in yoghurt made with ABT-1 starter culture and LB 2501.
[b] LA counts in yoghurt made with ABT-1 starter culture and LB 2501.
[c] BB counts in yoghurt made with ABT-1 starter culture and LB 2501.
[d] ST counts in yoghurt made with ABT-1 starter culture and LB 2515.
[e] LA counts in yoghurt made with ABT-1 starter culture and LB 2515.
[f] BB counts in yoghurt made with ABT-1 starter culture and LB 2515.

Table 4
Changes in viable counts of *S. thermophilus*, *L. delbrueckii* subsp. *bulgaricus*, *L. acidophilus* and *Bifidobacterium* sp. in ABT-4 starter cultures during manufacture and storage of yoghurt (ABT = *L. acidophilus*, *Bifidobacterium* sp. and *S. thermophilus*; ST = *S. thermophilus*; LA = *L. acidophilus*; BB = *Bifidobacterium* sp.; LB = *L. delbrueckii* subsp. *bulgaricus*)

	0 h	24 h	Week 1	Week 2	Week 3	Week 4
ST-4	6.04±0.04	9.00±0.03	8.88±0.03	9.00±0.03	9.88±0.08	8.84±0.06
LA-4	5.95±0.00	7.17±0.01	7.20±0.02	6.66±0.06	4.87±0.01	4.25±0.01
BB-4	6.00±0.01	6.99±0.03	6.85±0.03	6.62±0.01	6.80±0.04	6.69±0.04
ST-4[a] (ABT-4 + LB 2501)	6.08±0.03	9.21±0.01	9.16±0.00	9.96±0.10	9.45±0.48	8.90±0.02
LB 2501	5.99±0.02	8.10±0.06	8.07±0.03	7.81±0.03	7.34±0.01	7.06±0.02
LA-4[b] (ABT-4 + LB2501)	5.97±0.01	7.18±0.01	5.91±0.03	4.27±0.09	3.67±0.04	3.17±0.02
BB-4[c] (ABT-4 + LB2501)	6.02±0.02	6.94±0.04	6.58±0.02	6.54±0.03	6.66±0.08	6.70±0.02
ST-4[d] (ABT-4 + LB 2515)	6.15±0.02	8.99±0.02	9.36±0.61	8.90±0.00	8.99±0.02	8.91±0.01
LB 2515	5.99±0.02	7.47±0.01	7.06±0.03	6.29±0.01	6.22±0.02	7.11±0.03
LA-4[e] (ABT-4 + LB2515)	5.95±0.01	7.18±0.02	7.20±0.02	5.09±0.01	3.91±0.03	3.88±0.03
BB-4[f] (ABT-4 + LB2515)	5.97±0.02	6.72±0.01	6.83±0.04	6.86±0.04	6.68±0.02	6.68±0.04

[a] ST counts in yoghurt made with ABT-4 starter culture and LB 2501.
[b] LA counts in yoghurt made with ABT-4 starter culture and LB 2501.
[c] BB counts in yoghurt made with ABT-4 starter culture and LB 2501.
[d] ST counts in yoghurt made with ABT-4 starter culture and LB 2515.
[e] LA counts in yoghurt made with ABT-4 starter culture and LB 2515.
[f] BB counts in yoghurt made with ABT-4 starter culture and LB 2515.

Table 5
Changes in viable counts of *S. thermophilus*, *L. delbrueckii* subsp. *bulgaricus*, *L. acidophilus* and *Bifidobacterium* sp. in mix starter cultures during manufacture and storage of yoghurt (mix starter culture = ST 2002, LA 2415, BB 20210 and LB 2501; ST = *S. thermophilus*; LA = *L. acidophilus*; BB = *Bifidobacterium* sp.; LB = *L. delbrueckii* subsp. *bulgaricus*)

	0 h	24 h	Week 1	Week 2	Week 3	Week 4
ST-mix	4.98±0.03	7.84±0.09	7.93±0.01	8.95±0.00	7.69±0.04	7.42±0.04
LA-mix	4.54±0.01	7.69±0.09	7.27±0.01	7.28±0.01	7.07±0.01	7.19±0.01
BB-mix	4.68±0.03	7.05±0.06	7.09±0.03	7.23±0.01	7.73±0.03	6.72±0.04
ST[a] (mix + LB 2501)	4.92±0.08	7.65±0.11	8.19±0.01	9.12±0.01	7.95±0.01	7.96±0.01
LB 2501	5.97±0.02	7.85±0.04	7.88±0.02	7.87±0.02	7.56±0.04	6.92±0.00
LA[b] (mix + LB 2501)	4.52±0.01	6.92±0.02	6.94±0.01	6.90±0.01	6.82±0.03	6.92±0.03
BB[c] (mix + LB 2501)	4.70±0.03	6.61±0.02	6.70±0.10	6.66±0.04	7.28±0.01	6.30±0.02
ST[d] (mix + LB 2515)	4.96±0.01	7.09±0.09	8.20±0.00	7.65±0.08	6.82±0.04	8.23±0.03
LB 2515	6.00±0.01	7.86±0.07	7.96±0.05	7.82±0.05	7.50±0.02	6.94±0.02
LA[e] (mix + LB 2515)	4.50± 0.02	6.87±0.02	6.80±0.02	6.80±0.02	6.20±0.01	6.94±0.01
BB[f] (mix + LB 2515)	4.90±0.01	6.68±0.09	6.74±0.03	6.60±0.07	7.29±0.01	6.33±0.03

[a] ST counts in yoghurt made with mix starter culture and LB 2501.
[b] LA counts in yoghurt made with mix starter culture and LB 2501.
[c] BB counts in yoghurt made with mix starter culture and LB 2501.
[d] ST counts in yoghurt made with mix starter culture and LB 2515.
[e] LA counts in yoghurt made with mix starter culture and LB 2515.
[f] BB counts in yoghurt made with mix starter culture and LB 2515.

4. Conclusions

Supplementation of proteolytic strain of *L. delbrueckii* subsp. *bulgaricus* to ABT starter cultures reduced fermentation time for making yoghurt and increased firmness. All starter culture combinations examined produced EPS but differences in viscosity did not correlate with the amount of EPS produced. There were no significant differences in EPS production between the cultures studied suggesting that use of proteolytic strain of *L. delbrueckii* subsp. *bulgaricus* did not influence EPS production. Viability of probiotic bacteria was best in the yoghurt made with mix starter culture with the added *L. delbrueckii* subsp. *bulgaricus* followed by that with ABT-4 and ABT-1 starter cultures.

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